HYPERHOMOCYSTEINEMIA:

INHERITED CAUSES AND EFFECTS OF TREATMENT

DIANA GABRIËLLE FRANKEN

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Aan mijn ouders
Aan Geert

AAA = amino acid analyzer
CHD = coronary heart disease
CS = cystathionine synthase
CVD = cerebral vascular disease
DMSO = dimethylsulfoxide
DTE = dithioerythritol
DTT = dithiotreitol
DU = duplex measurements
FAD = flavin adenine dinucleotide
FOV = field of view
g2D-TOF MRA = gated 2D-time of flight magnetic resonance angiography
gT1 MRI = gated T1-weighted magnetic resonance imaging
HPLC = High Performance Liquid Chromatography
iaDSA = intraarterial digital subtraction angiography
MAT = methionine adenosyltransferase
mBrB = monobromobimane
methyl-THF = 5-methyltetrahydrofolate
mHH = mild hyperhomocysteinemia
MRA = magnetic resonance angiography
MRI = magnetic resonance imaging
MTHF = 5,10-methylenetetrahydrofolate
MTHFR = 5,10-methylenetetrahydrofolate reductase
MTOB = 4-methylthio-2-oxobutyrate
3-MTP = 3-methylthiopropionate
NaBH4 = sodiumborohydride
NE = not evaluable
NO = nitric oxide
PC = protein C
PVD = peripheral vascular disease
SAS = anterior spinal arterial syndrome
SBD-F = ammonium 7-fluorobenzo-2-oxa-1,3-diazide-4-sulphonate
SBM = segmental blood pressure measurement
TBP = tributylphosphine
TE = echo time
THF = tetrahydrofolate
TM = thrombomodulin
tPA = tissue-type plasminogen activator
TR = repetition time
vWF = von Willebrand factor
WHO = World Health Organization

General introduction and objectives of this thesis

Cardiovascular disease, in particular coronary artery disease, may lead to sudden death, and is responsible for about 40% of total mortality in the Netherlands[1]. Patients with arteriosclerotic vascular disease, surviving their cerebral, peripheral or coronary attack, may suffer from precocious disability. In the Netherlands in 1993, more than 1,500,000 days of stay in hospital were on account of complications of vascular diseases[1]. Consequently, investigations into arteriosclerotic and thrombotic diseases should not only be aimed at preventing the mortality but also at decreasing the morbidity. Tobacco smoking, diabetes mellitus, elevated blood pressure, and elevated blood cholesterol levels are well known risk factors for arteriosclerosis. Immobilization, use of oral contraceptives, pregnancy, recent childbirth, protein C, protein S, or antithrombin III deficiencies, and activated protein C resistance are established risk factors for venous thrombosis.

In 1969, a causative connection between arteriosclerotic vascular disease and the inborn error of methionine metabolism *homocystinuria*, characterized by severe accumulation of homocysteine in blood and tissues, was made by McCully[2]. Later on, it was estabished, that if homocysteine-lowering treatment is withheld from patients with cystathionine ß-synthase deficiency, the main cause of homocystinuria, about 30% will suffer from arterial or venous vascular disease before the age of 20 years and about 50% before the age of 30 years[3]. Since the initial observation of Wilcken and Wilcken[4] in 1976 that premature arteriosclerosis could probably be on the basis of mildly elevated blood homocysteine concentrations, the association of such *mild hyperhomocysteinemia*, with increased risk for cardiovascular disease has been scrutinized. During the last 20 years, various studies, i.e. case-control, cross-sectional, and prospective epidemiological studies have shown an increased prevalence of mild hyperhomocysteinemia among patients with arterial vascular disease. Nowadays, mild hyperhomocysteinemia is a generally accepted risk factor for cardiovascular disease. Furthermore, again in line with observations made in homocystinuria, recent studies also prove that mildly elevated plasma homocysteine concentrations are a risk factor for venous thrombosis as well.

Objectives of this thesis

Three main objectives of this thesis are to explore:

- firstly, what is the origin of mild hyperhomocysteinemia in vascular patients, and what are the prevalences of genetic causes?
- secondly, can vitamin supplementation lower or even normalize elevated homocysteine concentrations in such patients?
- and thirdly, will homocysteine-lowering treatment result in a clinically beneficial effect?

At the time the studies for this thesis were initiated, heterozygosity for homocystinuria was reported to account for almost all cases of mild hyperhomocysteinemia among vascular patients[5,6]. However, another enzyme defect eventually leading to hyperhomocysteinemia, i.e. homozygosity for a thermolabile mutant variant of methylenetetrahydrofolate reductase (MTHFR), was described already in 1991 by Kang et al.[7,8].

Previous studies among family members of arteriosclerotic hyperhomocysteinemic patients, to investigate the possible inheritance of mild hyperhomocysteinemia, included family members of coronary patients, but not those of patients with peripheral and cerebral arterial occlusive disease[9 11]. Apart from that, only fasting homocysteine levels have been measured, and not levels after standardized methionine loading in order to stress the metabolic pathway, as criterion of hyperhomocysteinemia.

Until 1991, the effect of homocysteine-lowering treatment in vascular patients was only reported sporadically. Boers et al.[12] presented data on normalization of the homocysteine concentration after methionine loading in 26 out of 32 vascular patients with post-load hyperhomocysteinemia by vitamin B_6 treatment. Brattstrom et al.[13] showed a 26% mean post-load increase lowering by vitamin B_6, and 39% by combined vitamin B_6 and folic acid supplementation. In contrast to vitamin B_6 solely, which showed no lowering of elevated fasting homocysteine concentrations, combined vitamin B_6 and folic acid led to a 53% decrease of the fasting homocysteine level However, in that report the duration of treatment, i e. two weeks, may have been to short to evaluate sufficiently such effect. Clinical effects of homocysteine-lowering treatment have not been reported.

The studies in this thesis are dealing with possible inherited causes of mild hyperhomocysteinemia in vascular patients (Part 1, Chapters 4 to 6), and with biochemical (Part 2, Chapters 7 to 10) and clinical (Part 3, Chapters 11 to 13) effects of homocysteine-lowering treatment in such patients. First an update of hyperhomocysteinemia until now is presented in **Chapter 2** and a comparison of three different laboratory determinations of blood homocysteine is described in **Chapter 3.**

In **Part 1,** the prevalence of mild hyperhomocysteinemia among family members of hyperhomocysteinemic vascular patients is explored in **Chapter 4.** We studied fasting and post-load homocysteine levels among 96 family members of 21 post-load hyperhomocysteinemic vascular index patients

The determination of thermolabile mutant variant of MTHFR and its prevalence among hyperhomocysteinemic patients with premature vascular disease is reported in **Chapter 5.**

In **Chapter 6,** a family with two inherited risk factors for vascular disease, i e mild hyperhomocysteinemia and protein C deficiency type 1, is described.

In **Part 2** of this thesis the biochemical effect of homocysteine-lowering treatment is demonstrated.

Chapter 7 gives a review of data on homocysteine-lowering treatment until 1996

Biochemical effects of vitamin B_6 and combined vitamin B_6 and folic acid supplementation in post-load hyperhomocysteinemic vascular patients are described in **Chapter 8** and **Chapter 9,** respectively.

In **Chapter 10,** the results of administration of vitamin B_1 in nine homozygotes for homocystinuria, who persisted to have elevated homocysteine levels in spite of conventional homocysteine-lowering treatment, are presented

Part 3 of this thesis is focused on the possible clinical effects of homocysteine-lowering treatment.

Chapter 11 presents findings on three markers of endothelial function, i.e. von Willebrand factor, thrombomodulin, and tissue-type plasminogen activator, in patients with peripheral arterial occlusive disease and mild hyperhomocysteinemia, before start and after one year of combined vitamin B_6 and folic acid therapy.

The reliability and accuracy of Magnetic Resonance Imaging and Magnetic Resonance Angiography examinations in the detection of mild arteriosclerosis in vascular patients with mild hyperhomocysteinemia are described in **Chapter 12**. The results justified the use of these non-invasive techniques in the prospective intervention study as described in **Chapter 13**. In a placebo-controlled prospective way of combined vitamin B_6 and folic acid therapy during 2 years, a possible clinical effect in vascular patients with mild hyperhomocysteinemia was investigated as a pilot study.

Finally, in **Chapter 14**, the presented studies are discussed, and several remarks are made about future perspectives.

References

1. Dutch Heart Foundation. Heart and vascular diseases in the Netherlands. Morbidity and mortality statistics. The Hague: Dutch Heart Foundation, (Dutch). 1995.
2. McCully KS. Vascular pathology of homocysteinemia: implications for the pathogenesis of arteriosclerosis. Am J Pathol 1969;56:111-128.
3. Mudd SH, Skovby F, Levy HL, Pettigrew KD, Wilcken B, Pyeritz RE, Andria G, Boers GHJ, Bromberg IL, Cerone R, Fowler B, Gröbe H, Schmidt H, Schweitzer L. The natural history of homocystinuria due to cystathionine-ß-synthase deficiency. Am J Hum Genet 1985;37:1-31.
4. Wilcken DEL and Wilcken B. The pathogenesis of coronary artery disease. A possible role for methionine metabolism. J Clin Invest 1976;57:211-215.
5. Boers GHJ, Smals AGH, Trijbels JMF, Fowler B, Bakkeren JAJM, Schoonderwaldt HC, Kleijer WJ, Kloppenborg PWC. Heterozygosity for homocystinuria in premature peripheral and cerebral occlusive arterial disease. N Engl J Med 1985;313:709-715.
6. Clarke R, Daly L, Robinson K, Naughten E, Cahalane S, Fowler B, Graham I. Hyperhomocysteinemia: an independent risk factor for vascular disease. N Engl J Med 1991;324:1149-1155.
7. Kang SS, Wong PWK, Bock HGO, Horwitz A, Grix A. Intermediate hyperhomocyteinemia resulting from compound heterozygosity of methylenetetrahydrofolate reductase mutations. Am J Hum Genet 1991;48:546-551.
8. Kang SS, Wong PWK, Susmano A, Sora J, Norusis M, Ruggie N. Thermolabile methylenetetrahydrofolate reductase: an inherited risk factor for coronary artery disease. Am J Hum Genet 1991;48:536-545.
9. Genest JJ, McNamara JR, Upson B, Salem DN, Ordovas JM, Schaefer EJ, Malinow MR. Prevalence of familial hyperhomocyst(e)inemia in men with premature coronary artery disease. Arterioscler Thromb 1991;11:1129-1136.
10. Wu LL, Wu J, Hunt SC, James BC, Vincent GM, Williams RR, Hopkins PN. Plasma homocyst(e)ine as a risk factor for early familial coronary artery disease. Clin Chem 1994;40:552-561.
11. Williams RR, Malinow MR, Hunt SC, Upson B, Wu LL, Hopkins PN, Stults BN, Kuida H. Hyperhomocyst(e)inemia in Utah siblings with early coronary disease. Coron Art Dis 1990;1:681-685.
12. Boers GHJ. The clinical usefulness of homocysteine determinations. In: Smith U, Eriksson S, Lingarde F (eds). Genetic susceptibility to environmental factors a challenge for public intervention. Almqvist, Wiksell International Inc., Stockholm, 1988;35-42.
13. Brattström L, Israelsson B, Norrving B, Bergqvist D, Thörne J, Hultberg B, Hamfelt A. Impaired homocysteine metabolism in early-onset cerebral and peripheral occlusive arterial disease. Effects of pyridoxine and folic acid treatment. Atheroscler 1990;81:51-60.

Hyperhomocysteinemia: an update

Homocysteine metabolism

The only source of homocysteine in men is demethylation of methionine, an essential amino acid. Via this pathway, up to 20 mmol of homocysteine is formed daily[1]. Homocysteine may either be transsulphurated or remethylated (Figure 2.1). The former pathway includes the conversion of homocysteine to cystathionine, catalyzed by the enzyme cystathionine ß-synthase. This enzyme requires a biologic active form of vitamin B_6, i e pyridoxal 5'-phosphate, as a cofactor The next step is the cleavage of cystathionine into homoserine and cysteine by γ-cystathionase, also a pyridoxal 5'-phosphate dependent enzyme. In the remethylation pathway, homocysteine is converted into methionine by either the enzyme betaine-homocysteine methyltransferase or by the enzyme methionine synthase. The former enzyme needs betaine (trimethylglycine) to donate a methylgroup to homocysteine. This enzyme is in human only present in the liver. Methionine synthase, active in most body tissues, requires vitamin B_{12} as a cofactor and 5-methyltetrahydrofolate (methyl-THF) as a substrate. Methyl-THF is formed from 5,10-methylenetetrahydrofolate which reaction is catalyzed by the enzyme 5,10-methylenetetrahydrofolate reductase (MTHFR) Thus, the enzymes involved in the metabolism of homocysteine are dependent on vitamin B_6, vitamin B_{12} and folate, which is also known as vitamin B_{11}

Transamination of methionine

Apart from transmethylation and transsulphuration, methionine can also be transaminated into 4-methylthio-2-oxobutyrate (MTOB), followed by oxydative decarboxylation of MTOB into 3-methylthiopropionate (3-MTP)[2 3] (Figure 2.1) The branched-chain 2-oxo acid dehydrogenase complex catalyzes this latter reaction which is supposedly the rate-limiting step in the transamination of methionine[3]. This complex requires the active form of vitamin B_1, thiamine pyrophosphate, as a cofactor 3-MTP is converted into methanethiol, dimethylsulphides, and methanethiol mixed disulphides Methionine degradation via the transamination pathway occurs in humans[4], but is probably of minor quantitative importance even in cystathionine ß-synthase deficient patients, despite their elevated methionine levels in blood[5] Only very recently, it has been reported that transamination in hypermethioninemic children is increased only if plasma methionine levels exceeded 300 or 350 μM[6 7].

Homocysteine terminology and methodology

In blood, about 70% to 80% of homocysteine is bound to protein The remaining non-protein bound homocysteine is present as homocystine (disulphide of homocysteine), or as homocysteine-cysteine mixed disulphide or as homocysteine The term free homocysteine refers in general to the non-protein bound homocysteine fractions, whereas total homocysteine refers to the sum of both, free plus protein bound forms of homocysteine.

The determination of the free homocysteine concentration in plasma has been described previously[8 9] Three methods for measuring total homocysteine concentration in plasma are described and compared with each other in Chapter 3[10] Homocysteine levels are often determined in the fasting state, but also during

the day ("basal" levels). Methionine-homocysteine metabolism can be stressed by oral loading with L-methionine (0.1 g/kg body weight). In such tests, blood homocysteine concentrations are preferentially measured at peak post-load homocysteine levels, which occurs between 4 to 8 hours after the methionine load[11,12]. For practical reasons only one post-load blood sample, mostly that of six hours after loading, is drawn.

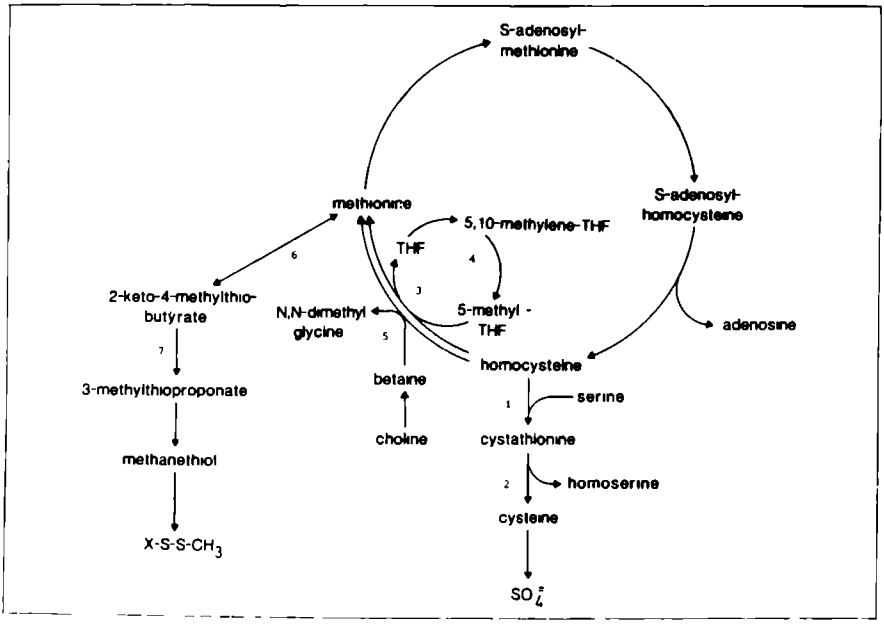

Figure 2.1. The methionine metabolism. 1 = cystathionine β-synthase, 2 = γ-cysthationase, 3 = methionine synthase, 4 = 5,10-methylenetetrahydrofolate reductase, 5 = betaine-homocysteine methyltransferase , 6 = transaminase, 7 = branched-chain 2-oxo acid dehydrogenase.

Causes of severe hyperhomocysteinemia

The classic and most common form of severe hyperhomocysteinemia, also known as homocystinuria, is caused by cystathionine ß-synthase deficiency[1]. Far over 600 patients of such homozygotes for homocystinuria have been described[13]. Deficient activity of the enzymes MTHFR, methionine synthase, or inborn errors of methylcobalamin synthesis are other conditions leading to severe hyperhomocysteinemia[1]. These latter three conditions are very rare, with reports in the literature of about 50 patients[14-20].

Causes of mild hyperhomocysteinemia

Heterozygotes for homocystinuria have reduced activity of cystathionine ß-synthase of about 20% of normal activity[1,9,21]. These subjects have elevated post-methionine load plasma homocysteine concentrations, with normal or slightly elevated fasting homocysteine levels[9,22]. The incidence of homozygous cystathionine ß-synthase deficiency is estimated to be approximately 1:344,000 live births worldwide, but may be as high as 1:58,000 in Ireland[1]. In the Netherlands, the exact incidence of homozygotes is unknown because neonatal screening of this specific inborn error is not being carried out. Homocystinuria is inherited in an autosomal recessive fashion, and so the incidence of the heterozygous cystathionine ß-synthase deficiency can be calculated to be about 1:293 worldwide and as high as 1:125 in the Irish population[1,23]. Boers et al.[24] and six years later Clarke et al.[25] reported that heterozygosity for homocystinuria, in the Netherlands and Ireland, respectively, accounted for the hyperhomocysteinemia in almost all their detected vascular patients. However, from a large questionnaire study among 203 families with homozygotes for homocystinuria, Mudd et al.[26] estimated that no more than 5% of the heterozygous family members might have coronary vascular disease, with a relative risk of the fathers of homocystinuric patients of 1.43 (95% confidence interval 0.5 - 3.7), so not statistically significantly increased. The frequency of carriers for homocystinuria is by far not as large as the frequency of mild hyperhomocysteinemia which is in the normal population about 5% to 8%[27,28] or to account for the number of vascular patients with mild hyperhomocysteinemia[23]. Therefore, heterozygosity for homocystinuria can not be responsible for all cases of mild hyperhomocysteinemia, and the observation by Engbersen et al.[21] (Chapter 5) is in line with this conclusion. Furthermore, Kluijtmans et al.[30] reported that heterozygosity for the 833 T→C mutation in cystathionine ß-synthase, which in the homozygous form is found in half of the alleles in Dutch homozygous cystathionine ß-synthase deficient patients, was found in not any of the 60 studied patients with premature vascular disease, and only in one of the 111 control subjects. The same holds true for the Irish mutation 919 G→A, which could not be detected in 100 Irish patients with premature arteriosclerotic disease[29]. Kozich et al.[31] was not able to identify any mutation in the cystathionine ß-synthase cDNA in 4 vascular patients with mild hyperhomocysteinemia.

Other genetic or non-genetic causes must account for the majority of cases of mild hyperhomocysteinemia. Blood homocysteine concentrations proved to be correlated in healthy twins without vascular disease[32,33] and among family members of patients with coronary[34-36], various vascular arterial occlusive disease[37] or juvenile venous thrombosis[38]. Therefore, mild hyperhomocysteinemia can be

concluded to be genetically determined at least in a part of the cases. Kang et al.[14,39,40] reported an increased incidence of thermolabile variant of MTHFR deficiency among patients with coronary vascular disease. These patients have a specific activity of approximately 50% of normal, and a residual activity after heat inactivation of less than 30% compared to 50% residual activity in control subjects. Such patients may have normal or elevated homocysteine concentrations[14,39,40]. This thermolabile MTHFR deficiency is inherited autosomal recessively[39]. Engbersen et al.[21] found thermolabile MTHFR deficiency in 28% of the hyperhomocysteinemic patients with premature vascular disease. Very recently, homozygous 677 C→T mutation in the MTHFR gene is shown to cause this thermolabile variant of MTHFR[41]. This mutation in homozygous form could be determined three times more frequent in vascular patients than in control individuals, 15% and 5.4%, respectively, which constitutes a relative risk of this genetic condition for cardiovascular disease of 3.1 (95% confidence interval of 1.0 to 9.2)[30]. Compound heterozygotes for MTHFR deficiency with one allele for the severe form of MTHFR and one allele for the thermolabile form are mostly hyperhomocysteinemic[14]. Non-genetic causes, such as chronic renal failure, liver disease, nutritional or pharmacological induced deficiencies of folate, vitamin B_{12}, or vitamin B_6, can also account for increased homocysteine concentrations as reviewed extensively by Ueland et al.[12,38] and Refsum et al.[42].

Hyperhomocysteinemia and cardiovascular disease

The Australians Wilcken and Wilcken[43] reported in 1976 that 7 out of 25 patients, with angiographically documented ischemic heart disease at ages younger than 50 years, had higher homocysteine levels after methionine load than matched control subjects. The association between mild hyperhomocysteinemia and several forms of premature arteriosclerosis, i.e. cerebral, peripheral and coronary, have been determined by Boers et al.[24,44] and Brattström et al.[45], and many studies published since then consolidated these findings. In pooled data from separate reports, it is calculated that the prevalence of mild hyperhomocysteinemia in patients with peripheral, i.e. 32% to 40%, and cerebral arterial occlusive disease, i.e. 24% to 25%, may be higher than in patients with coronary arterial occlusive disease, i.e. 15% to 21%[27,46,47]. The percentages given in the studies presented in Chapters 8 and 9 were well in line with these summarized data[11,48].

Most reports were of retrospective nature, such as case-control or cross-sectional studies. Six, however, have been conducted prospectively and three of those showed results compatible with those of the retrospective studies, so supporting the association between hyperhomocysteinemia and cardiovascular disease[49-51]. However, three other prospective studies among patients with ischemic stroke[52,53], myocardial infarction[53], and severe angina pectoris[54] revealed no significant, albeit slightly elevated fasting homocysteine blood levels in patients compared to controls.

Since recently, evidence has been accumulated that mild hyperhomocysteinemia is also a risk factor for venous thrombosis[38,55-58]. This relationship was found in patients with primary or recurrent venous thrombosis, and at all ages. Brattström et al.[59], and recently also Amundsen et al.[60] found no significant difference in plasma homocysteine concentration, between small groups

of patients and controls. Mild hyperhomocysteinemia has further on been related to the occurrence of retinal artery or vein occlusion[55,61].

The relationship between the plasma homocysteine concentration and severity of the arteriosclerosis, in peripheral[62,63], cerebral[54], and coronary[64,65] arteries, is more likely to be graded than indicative of a threshold effect. Thus, the higher the plasma homocysteine concentration within a patient, the higher the risk of arteriosclerosis. Such a graded relationship with homocysteine blood levels was also found in asymptomatic subjects as much as carotic stenosis concerns[66,67]. On the other hand, den Heijer et al.[68] reported, in patients with venous thrombosis, an increased risk only above a certain homocysteine level, which finding suggests a threshold relationship between homocysteine concentration and risk for thrombosis.

Pathogenesis of arteriosclerosis

Although the relationship between hyperhomocysteinemia and arteriosclerosis has been extensively reported, the underlying mechanism(s) of mild hyperhomocysteinemia in causing vascular damage has not been elucidated until now. Effects of homocysteine such as:

1. damaging endothelial cells and proliferation of smooth muscle cells in arterial vessel walls,
2. activation of blood clotting factors causing fibrin formation, and
3. increased platelet adhesion and aggregation at the side of the arterial intimal damage have been described.

Effect on endothelium

Harker et al.[68] described, in an animal model, sustained endothelial cell injury by homocysteine infusion, leading to desquamation of endothelial cells which initiates development of arteriosclerotic lesions. They hypothesized that arteriosclerosis in hyperhomocysteinemia is based on this directly damaging effect of the highly reactive sulfhydryl-containing amino acid. The concentration of homocyst(e)ine used was, however, even higher than that observed in homozygotes for homocystinuria. In vitro, the lowest concentration of homocysteine in its reduced form resulting in endothelial cell loss was 60 μmol/L[69]. Still, in patients with mild hyperhomocysteinemia with fasting total homocysteine levels up to maximally 50 μmol/L, the concentration of homocysteine in plasma is much lower considering the fact that only about 1% of the total amount of blood homocysteine is present in its reduced non-protein bound state[70,71]. So, the relevance of these studies for the in vivo situation in mild hyperhomocysteinemia is questionable.

Oxidation of thiol-groups of amino acids, such as homocysteine, can generate reactive oxygen radicals[72,73]. In vitro studies showed that homocysteine-induced endothelial cell injuries might be on the basis of hydrogen peroxide (H_2O_2)[72] which is generated by oxidation of homocysteine to its disulphides. H_2O_2 may also increase the level of oxidized low-density lipoproteins[72]. However, these findings could not be reproduced by others[74-76]. A recent in vitro study provided evidence that at physiological concentrations, homocysteine reacts with nitric oxide (NO) to form S-nitroso-homocysteine, which potentiates the vasodilatory and antiplatelets effect of NO[77]. Concomitantly, by this reaction the pathogenicity of homocysteine

is supposed to be attenuated because of inhibition of its sulphhydryl group's oxidation If the concentration of homocysteine exaggerates that of NO, this part of homocysteine is not inactivated anymore and may carry out its toxicity to endothelial cells. The following resulting endothelial cell loss affects NO synthesis itself, by which a cycle is started resulting in arteriosclerosis and thrombosis[77].

Besides affecting endothelium, homocysteine can reportedly induce proliferation of smooth-muscle cells, a key feature of arteriosclerosis[78].

Effect on coagulation proteins

Endothelial cells produce various proteins of the coagulation and fibrinolytic system, such as thrombomodulin, von Willebrand factor, and tissue-type plasminogen activator Elevated levels of these proteins in plasma may indicate ongoing endothelial injuries[79 81]. The cardiovascular prognosis in survivors of myocardial infarction with high plasma von Willebrand factor levels has been shown to be poorer than in those with normal concentrations[79]. A in vivo study by van den Berg et al.[82 83] in mildly hyperhomocysteinemic patients with peripheral arterial occlusive disease showed indeed elevated levels of plasma von Willebrand factor and thrombomodulin. Chapter 11 presents data on amelioration of these markers of endothelial dysfunction by homocysteine-lowering treatment[83]

Thrombomodulin is an anticoagulant protein on the endothelial surface, which serves as a cofactor for the activation of protein C by thrombin. Deficiency of the anticoagulant protein C is an independent risk factor for venous and arterial thrombosis[84 85]. In vitro studies showed that homocysteine inhibits thrombomodulin expression on endothelial cell surface, and thereby hampers protein C coactivation[86 88]. It also inactivates protein C irreversibly[86 88], resulting in a tendency to venous and arterial thrombosis. This inhibition of thrombomodulin expression on cell membrane by homocysteine in in vitro studies seems to be in contradiction with the in vivo findings that plasma thrombomodulin concentration is elevated in vascular patients with mild hyperhomocysteinemia[82 83]. This contradiction may be explained by the use of enormously high concentration of reduced homocysteine in in vitro studies compared to this homocysteine concentration in vivo. Furthermore, apart from inhibition of thrombomodulin expression on the endothelial cells, homocysteine is reported to rapidly reduce activated protein C generation with a 50% decrease of the activity within half an hour after exposure to homocysteine[86 88]. This effect is probably due to competitive inhibition by homocysteine of the thrombin-thrombomodulin interaction.

In this respect, it is very interesting that in one family with combined mild hyperhomocysteinemia and protein C deficiency, only those family members with both inherited risk factors for arterial and venous thrombosis manifested with vascular disease[89]. In line with this observation, the report of Mandel et al.[90] is remarkable. In seven families with at least one homozygote for homocystinuria, venous or arterial thrombosis in these homozygotes exclusively occurred only in the presence of factor V Leiden mutation (either in the heterozygous or homozygous form), which leads to resistance of activated protein C[90] These reports suggest that hyperhomocysteinemia and deficiency of activated protein C or resistance against it, are acting synergistically in inducing arteriosclerosis and/or thrombosis In another very recent study by den Heijer et al [58], however, it was not possible to establish a synergism of mild hyperhomocysteinemia and factor V Leiden mutation

in excess risk of thrombosis, probably because of insufficient number of included cases with both conditions combined.

In patients with homocystinuria, with severely elevated homocysteine levels, reduced antithrombin III activity and factor VII deficiency have been demonstrated which normalized during homocysteine-lowering treatment[91-93]. However, another study could not confirm reduced antithrombin III levels in patients with hyperhomocysteinemia due to vitamin B_{12} or folate deficiency[94]. Rodgers and Kane[95] reported that homocysteine increases endothelial cell factor V activity.

Effect on platelets

Normally, platelets ignore vascular endothelium, but once the vessel wall is damaged, platelet adhesion occurs. Platelet counts were not affected in four studied homozygotes for homocystinuria, but their platelet survival was reduced by half, which improved and almost normalized after start with vitamin B_6 treatment[69]. On the other hand, other studies reported normal platelet survival in such patients[96,97].

In conclusion, although abnormalities of endothelial cells, coagulation factors, platelets or disorders in the complex interaction of these factors have been held responsible for the vascular events in hyperhomocysteinemia, the underlying mechanism is far from elucidated at the moment.

References

1. Mudd SH, Levy HL, Skovby F. Disorders of transsulfuration. In: Scriver CR, Beaudet AL, Sly WS, Valle D (eds). The metabolic basis of inherited disease. McGraw-Hill Inc., New York, 1995;1279-1327.
2. Blom HJ, van den Elzen JPAM, Yap SH, Tangerman A. Methaniol and dimethylsulfide formation from 3-methylthiopropionate in human and rat hepatocytes. Biochem Biophys Acta 1988;972:131-136.
3. Blom HJ, Boers GHJ, van den Elzen JPAM, Gahl WA, Tangerman A. Transamination of methionine in humans. Clin Sci 1989;76:43-49.
4. Jones MA, Yeaman SJ. Oxidative decraboxylation of 4-methylthio-2-oxobutyrate by branched-chain 2-oxo acid dehydrogenase complex. Biochem J 1986;237:621-623.
5. Blom HJ, Boers GHJ, van Roessel JJM, Trijbels JMF, Tangerman A. Cystathionine synthase deficient patients do not utilize the transamination pathway of methionine to control hypermethioninemia and homocysteinemia. Metabolism 1989;38:577-582.
6. Tangerman A, Mudd SH, Levy HL. In: Mato JM, Caballero A (eds). Methionine metabolism: molecular mechanisms and clinical implications. Bouncopy Inc., Madrid, 1994;211-216.
7. Mudd SH, Levy HL, Tangerman A, Boujet C, Buist N, Davidson-Mundt A, Hudgins L, Oyanagi K, Nagao M, Wilson WG. Isolated persistent hypermethionemia. Am J Hum Gen 1995;57:882-892.
8. Boers GHJ, Smals AGH, Trijbels JMF, Leermakers AI, Kloppenborg PWC. Unique efficiency of methionine metabolism in premenopausal women may protect against vascular disease in the reproductive years. J Clin Invest 1983;72:1971-1976.
9. Boers GHJ, Fowler B, Smals AGH, Trijbels JMF, Leermakers AI, Kleijer WJ, Kloppenborg PWC. Improved identification of heterozygotes for homocystinuria due to cystathionine synthase deficiency by the combination of methionine loading and enzyme determination in cultured fibroblasts. Hum Genet 1985;69:164-169.
10. te Poele-Pothoff MTWB,, van den Berg M, Franken DG, Boers GHJ, Jakobs C, de Kroon IFI, Eskes TKAB, Trijbels JMF, Blom HJ. Three different methods for the determination of total homocysteine in plasma. Annals of Clin Biochem 1995;32:218-220.
11. Franken DG, Boers GHJ, Blom HJ, Trijbels JMF, Kloppenborg PWC. Treatment of mild hyperhomocysteinemia in vascular disease patients. Arterioscler Thromb 1994;14;465-470.
12. Ueland PM, Refsum H. Plasma homocysteine, a risk factor for vascular disease: plasma levels in health, disease, and drug therapy. J Lab Clin Med 1989;473-501.
13. Mudd SH, Skovby F, Levy HL, Pettigrew KD, Wilcken B, Pyeritz RE, Andria G, Boers GHJ, Bromberg IL, Cerone R, Fowler B, Gröbe H, Schmidt H, Schweitzer L. The natural history of homocystinuria due to cystathionine-ß-synthase deficiency. Am J Hum Genet 1985;37:1-31.
14. Kang SS, Wong PWK, Bock HGO, Horwitz A, Grix A. Intermediate hyperhomocyteinemia resulting from compound heterozygosity of methylenetetrahydrofolate reductase mutations. Am J Hum Genet 1991;48:546-551.
15. Visy JM, Le Coz P, Chadefaux B, Fressinaud C, Woimant F, Marquet J, Zittoun J, Visy J, Vallat JM, Haguenau M. Homocystinuria due to 5,10-methylenetetrahydrofolate reductase deficiency revealed by stroke in adult siblings. Neurology 1991;41:1313-1315.
16. Mudd SH, Uhlendorf BW, Freeman JM, Finkelstein JD, Shih VE. Homocystinuria associated with decreased methylenetetrahydrofolate reductase activity. Biochem Biophys Res Commun 1972;46:905-912.
17. Erbe RW. Inborn errors of folate metabolism. In: Blakely RL (ed). Folates and

pterines. John Wiley & sons Inc., Boston, 1986:413-465.
18. Cooper BA, Rosenblatt DS. Inherited defects of vitamin B_{12} metabolism. Annu Rev Nutr 1987;7:291-320.
19. Hallam LJ, Sawyer M, Clarke ACL, van der Weyden MB. Vitamin B_{12}-responsive neonatal megaloblastic anemia and homocystinuria with associated reduced methionine synthase activity. Blood 1987;69:1128-1133.
20. Schuh S, Rosenblatt DS, Cooper BA, Schroeder M-L, Bishop AJ, Seargeant LE, Haworth JC. Homocystinuria and megaloblastic anemia reponsive to vitamin B_{12} therapy. An inborn error of metabolism due to defect in cobalamin metabolism. N Eng J Med 1984;310:686-690.
21. Engbersen AMT, Blom HJ, Franken DG, Boers GHJ, Stevens EMB, Poirot CM, Trijbels JMF. Thermolabile 5,10-methylenetetrahydrofolate reductase as a cause of mild hyperhomocysteinemia. Am J Hum Genet 1995;56:142-150.
22. McGill JJ, Mettler G, Rosenblatt DS, Scriver CR. Detection of heterozygotes for recessive alleles. Homocyst(e)inemia: paradigm of pitfalls in phenotypes. Am J of Med Genet 1990;36:45-52.
23. Daly L, Robinson K, Tan KS, Graham IM. Hyperhomocysteinemia: a metabolic risk factor for coronary heart disease determined by both genetic and environmental influences? Quart J Med 1993;86:685-689.
24. Boers GHJ, Smals AGH, Trijbels JMF, Fowler B, Bakkeren JAJM, Schoonderwaldt HC, Kleijer WJ, Kloppenborg PWC. Heterozygosity for homocystinuria in premature peripheral and cerebral occlusive arterial disease. N Engl J Med 1985;313:709-715.
25. Clarke R, Daly L, Robinson K, Naughten E, Cahalane S, Fowler B, Graham I. Hyperhomocysteinemia: an independent risk factor for vascular disease. N Engl J Med 1991;324:1149-1155.
26. Mudd SH, Havlik R, Levy HL, McKusick VA, Feinleib M. A Study of cardiovascular risk in heterozygotes for homocystinuria. Am J Hum Genet 1981;33:883-893.
27. Ueland PM, Refsum H, Brattström L. Plasma homocysteine and cardiovascular disease. In: Francis RBF. Atherosclerotic cardiovascular disease, hemostasis, and endothelial function. Marcel Dekker, Inc., New York, 1992;183-236.
28. Kang S-S, Wong PWK, Malinow MR. Hyperhomocysteinemia as a risk factor for occlusive vascular disease. Annu Rev Nutr 1992;12:279-298.
29. Whitehead AS, Ward P, Tan S, Naugthen E, Kraus JP, Sellar GC, McConell DJ et al. The molecular genetics of homocystinuria, hyperhomocysteinemia, and premature vascular disease in Ireland. In: Mato JM, Caballero A (eds). Methionine metabolism: molecular mechanism and clinical implications. Bouncopy Inc., Madrid, 1994;79-83.
30. Kluijtmans LAJ, van den Heuvel LPWJ, Boers GHJ, Frosst P, Stevens EMB, van Oost BA, den Heijer M, Trijbels JMF, Rozen R, Blom HJ. Molecular genetic analysis in mild hyperhomocysteinemia: a common mutation in the methylenetetrahydrofolate reductase gene is a genetic risk factor for cardiovascular disease. Am J Hum Genet 1996;58:35-41.
31. Kozich V, Kraus E, de Franchis R, Fowler B, Boers GHJ, Graham I, Kraus JP. Hyperhomocysteinemia in premature arterial disease: examination of cystathionine ß-synthase alleles at the molecular level. Hum Mol Genet 1995;4:623-629.
32. Reed T, Malinow MR, Christian JC, Upson B. Estimates of heritability of plasma homocyst(e)ine levels in aging adult male twins. Clin Genet 1991;39:425-428.
33. Berg K, Malinow MR, Kierulf P, Upson B. Population variation and genetics of plasma homocyst(e)ine level. Clin Genet 1992;41:315-321.
34. Williams RR, Malinow MR, Hunt SC, Upson B, Wu LL, Hopkins PN, Stults BN, Kuida H. Hyperhomocyst(e)inemia in Utah siblings with early coronary disease. Coron Art Dis 1990;1:681-685.
35. Genest JJ, McNamara JR, Upson B, Salem DN, Ordovas JM, Schaefer EJ,

Malinow MR. Prevalence of familial hyperhomocyst(e)inemia in men with premature coronary artery disease. Arterioscler Thromb 1991;11:1129-1136.
36. Wu LL, Wu J, Hunt SC, James BC, Vincent GM, Williams RR, Hopkins PN. Plasma homocyst(e)ine as a risk factor for early familial coronary artery disease. Clin Chem 1994;40:552-561.
37. Franken DG, Boers GHJ, Blom HJ, Cruysberg JRM, Trijbels JMF, Hamel BCJ. Prevalence of familial mild hyperhomocysteinemia. Atheroscler 1996;125:71-80.
38. Falcon CR, Cattaneo M, Panzeri D, Martinelli I, Mannucci PM. High prevalence of hyperhomocyst(e)inemia in patients with juvenile venous thrombosis. Arterioscler Thromb 1994;14:1080-1083.
39. Kang SS, Wong PWK, Susmano A, Sora J, Norusis M, Ruggie N. Thermolabile methylenetetrahydrofolate reductase: an inherited risk factor for coronary artery disease. Am J Hum Genet 1991;48.536-545.
40. Kang S-S, Passen EL, Ruggie N, Wong PWK, Sora H. Thermolabile defect of methylenetetrahydrofolate reductase in coronary artery disease. Circ 1993;88:1463-1469.
41 Frosst P, Blom HJ, Milos R, Goyette P, Sheppard CA, Matthews RG, Boers GHJ, den Heijer M, Kluijtmans LAJ, van den Heuvel LP, Rozen R. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. Nat Genet 1995;10:111-113.
42 Refsum H, Ueland PM. Clinical significance of pharmacological modulation of homocysteine metabolism. Trends Pharmacol Sci 1990;11:411-416.
43. Wilcken DEL and Wilcken B. The pathogenesis of coronary artery disease. A possible role for methionine metabolism. J Clin Invest 1976;57:211-215.
44. Boers GHJ, Schoonderwaldt HC, Schulte BPM, Trijbels JMF, Smals AGH, Kloppenborg PWC. Heterozygosity for homocystinuria: a risk factor of occlusive cerebrovascular disease? Clin Genet 1983;24:300-301.
45. Brattstrom LE, Hardebo JE, Hultberg BL. Moderate homocysteinemia - a possible risk factor for arteriosclerotic cerebrovascular disease. Stroke 1984;15·1012-1016.
46. Boers GHJ. Hyperhomocysteinaemia: a newly recognized risk factor for vascular disease. Neth J Med 1994;45:34-41.
47. Ueland PM, Refsum H, Stabler SP, Malinow MR, Andersson A, Allen RH. Total homocysteine in plasma or serum Methods and clinical applications. Clin Chem 1993;39:1764-1779.
48 van den Berg M, Franken DG, Boers GHJ, Blom HJ, Jakobs C, Rauwerda JA. Combined vitamin B_6 plus folic acid therapy in young patients with arteriosclerosis and hyperhomocysteinemia. J Vasc Surg 1994;20:933-940.
49 Stampfer MJ, Malinow MR, Willett WC, Newcomer LM, Upson B, Ullman D. A prospective study of plasma homocyst(e)ine and risk of myocardial infarction in US physicians. JAMA 1992;268:877-881.
50 Arnesen E, Refsum H, Bonaa KH, Ueland PM, Forde OH, Nordrehaug JE. Serum total homocysteine and coronary heart disease. Int J of Epidem 1995;24:704-709.
51 Perry IJ, Refsum H, Ebrahim SB, Ueland PM, Shaper AG. Prospective study of serum total homocysteine concentration and risk of stroke in middle-aged British men. Lancet 1995;346:1395-1398.
52. Verhoef P, Hennekens CH, Malinow MR, Kok FJ, Willett WC, Stampfer MJ. A prospective study of plasma total homocysteine and risk of ischemic stroke Stroke 1994;25:1924-1930.
53. Alfthan G, Pekkanen J, Jauhiainen M,Pitkaniemi J, Karvonen M, Tuomilehto J, Salonen JT, Ehnholm C. Relation of serum homocysteine and lipoprotein(a) concentrations to atherosclerotic disease in a prospective Finnish population based study. Atherosclerosis 1994;106:9-19.
54 Verhoef P, Hennekens CH, Allen RH, Stabler SP, Willett WC, Stampfer MJ. Plasma total homocysteine and future risk of angina pectoris with evidence

of severe coronary atherosclerosis. Submitted.
55. Bienvenu T, Ankri A, Chadefaux B, Kamoun P. Dosage de L'homocystéine plasmatique dans l'exploration des thromboses du sujet jeune. Presse Méd 1991;20:985-988.
56. den Heijer M, Blom HJ, Gerrits WBJ, Rosendaal FR, Haak HL, Wijermans PW, Bos GMJ. Is hyperhomocysteinemia a risk factor for recurrent venous thrombosis? Lancet 1995;345:882-885.
57. Fermo I, D'Angelo SV, Paroni R, Mazzola G, Calori G, D'Angelo A. Prevalence of moderate hyperhomocysteinemia in patients with early-onset venous and arterial occlusive disease. Ann Intern Med 1995;123:747-753.
58. den Heijer M, Koster T, Blom HJ, Bos GMJ, Briët E, Reitsma PH, Vandenbroucke JP, Rosendaal FR. Hyperhomocysteinemia as a risk factor for deep-vein thrombosis. N Eng J Med 1996;334:759-762.
59. Brattström L, Tengborn L, Lagerstedt C, Israelsson B, Hultberg B. Plasma homocysteine in venous thromboembolism. Haemostasis 1991;21:51-57.
60. Amundsen T, Ueland PM, Waage A. Plasma homocysteine levels in patients with deep venous thrombosis. Arterioscl Thromb Vasc Biol 1995;15:1321-1323.
61. Wenzler EM, Rademakers AJJM, Boers GHJ, Cruysberg JRM, Webers CAB, Deutman AF. Hyperhomocysteinemia in retinal artery and retinal vein occlusion. Am J Ophth 1993;115:162-167.
62. Taylor LM, DeFrang RD, Harris EJ, Porter JM. The association of elevated plasma homocyst(e)ine with progression of symptomatic peripheral arterial disease. J Vasc Surg 1991;13:128-136.
63. van den Berg M, Stehouwer CDA, Bierdrager E, Rauwerda JA. Plasma homocysteine and severity of atherosclerosis in young patients with lower extremity atherosclerotic disease. Atheroscler Thromb Vasc Biol 1996;16:165-171.
64. von Eckardstein A, Malinow MR, Upson B, Heinrich J, Schulte H, Schönfeld R, Köhler E, Assman G. Effects of age, lipoproteins, and hemostatic parameters on the role of homocyst(e)inemia as a cardiovascular risk factor in men. Arterioscler Thromb 1994;14:460-464.
65. Hopkins PN, Wu LL, Wu J, Hunt SC, James BC, Vincent GM, Williams RR. Higher plasma homocyst(e)ine and increased susceptibility to adverse effects of low folate in early familial coronary artery disease. Arterioscl Thromb Vasc Biol 1995;15:1314-1320.
66. Malinow MR, Nieto FJ, Szklo M, Chambless LE, Bond G. Carotid artery intimal-medial wall thickening and plasma homocyst(e)ine in asymptomatic adults. Circulation 1993;87:1107-1113.
67. Selhub J, Jacques PF, Bostom AG, D'Agostino RB, Wilson PWF, Belanger AJ, O'Leary DH, Wolf PA, Schaefer EJ, Rosenberg IH. Association between plasma homocysteine concentrations and extracranial carotid-artery stenosis. N Eng J Med 1995;332:286-291.
68. Harker LA, Slichter SJ, Scott CR, Ross R. Homocystinemia: Vascular injury and arterial thrombosis. N Eng J Med 1974;291:537-543.
69. Harker LA, Ross R, Slichter SJ, Scott CR. Homocystine-induced arterioslerosis: The role of endothelial cell injury and platelets response in its genesis. J Clin Invest 1976;58:731-741.
70. Mansoor MA, Guttormsen AB, Fiskerstrand T, Refsum H, Ueland PM, Svardal AM. Redox status and protein binding of plasma aminothiols during transient hyperhomocysteinemia that follows homocysteine administration. Clin Chem 1993;39:980-985.
71. Blom HJ, van der Molen EF. Pathobiochemical implications of hyperhomocysteinemia. Fibrinolysis 1994;8:86-87.
72. Starkebaum G, Harlan JM. Endothelial cell injury due to copper-catalyzed hydrogen peroxide generation from homocysteine. J Clin Invest 1986;77:1370-1376.

73. Heinecke JW, Rosen H, Suzuki LA, Chait A. The role of sulfur-containing amino acids in superoxide production and modification of low density lipoprotein by arterial smooth muscle cells. J Biol Chem 1987;262:10098-10103.
74. Dudman NPB, Hicks C, Wang J, Wilcken DEL. Human arterial endothelial cell detachment in vitro: its promotion by homocysteine and cysteine. Atheroscler 1991;91:77-83.
75. Dudman NPB, Wilcken DEL, Stocker R. Circulating lipid hydroperoxide levels in human hyperhomocysteinemia - Relevance to development of arteriosclerosis. Arterioscler Thromb 1993;13:512-516.
76. Blom HJ, Kleinveld HA, Boers GHJ, Demacker PNM, Hak - Lemmers HLM, te Poele - Pothoff MTWB, Trijbels JMF. Lipid peroxidation and susceptibility of low-density lipoprotein to in vitro oxidation in hyperhomocysteinemia. Eur J Clin Invest 1995;25:149-154.
77. Stamler JS, Osborne JA, Jaraki O, Rabbani LE, Mullins M, Singel D, Loscalzo J. Adverse vascular effects of homocysteine are modulated by endothelium-derived relaxing factor and related oxides of nitrogen. J Clin Invest 1993;91:308-318.
78. Tsai J-C, Perrella MA, Yoshizumi M, Hsieh C-M, Haber E, Schlegel R, Lee M-E. Promotion of vascular smooth muscle cell growth by homocysteine: a link to atherosclerosis. Proc Natl Acad Sci USA 1994;91:6369-6373.
79. Jansson JH, Nilsson TK, Johnson O. Von Willebrand factor in plasma: a novel risk factor for recurrent myocardial infarction and death. Br Heart J 1991;66:351-355.
80. Ishii H, Uchiyama H, Kazama M. Soluble thrombomodulin antigen in conditioned medium is increased by damage of endothelial cells. Thromb Haemostas 1991;65:618-623.
81. Seigneur M, Dufourcq P, Conri C, Constans J, Mercié P, Pruvost A, Amiral J, Midy D, Baste J-C, Boisseau MR. Levels of plasma thrombomodulin are increased in atheromatous arterial disease. Thromb Res 1993;71:423-431.
82. van den Berg M, Stehouwer CDA, Boers GHJ, Rauwerda JA, Kluft C. Arterial disease in hyperhomocysteinemia and the effect of treatment: a pilot study. Fybrinolysis 1994;8(2):88-90.
83. van den Berg M, Boers GHJ, Franken DG, Blom HJ, van Kamp GJ, Jacobs C, Rauwerda JA, Kluft C, Stehouwert CDA. Hyperhomocysteinemia and endothelial dysfunction in young patients with peripheral arterial occlusive disease. Eur J Clin Invest 1995;25:176-181.
84. Hedner U, Davie EW. Blood and blood-forming tissue. In: Scriver CR, Beaudet AL, Sly WS, Valle D (eds). The metabolic basis of inherited disease. McGraw-Hill Inc., New York, 1989;2107-2134.
85. Coller BS, Owen J, Jesty J, Horowitz D, Reitmann MJ, Spear J, Yeh T, Comp PC. Deficiency of plasma protein S, protein C or antithrombin III and arterial thrombosis. Arteriosclerosis 1987;7:456-461.
86. Lentz SR, Sadler JE. Inhibition of thrombomodulin surface expression and protein C activation by the thrombogenic agent homocysteine. J Clin Invest 1991;88:1906-1914.
87. Hayashi T, Honda G, Suzuki K. An atherogenic stimulus homocysteine inhibits cofactor activity of thrombomodulin and enhances thromomodulin expression in human umbilical vein endothelial cells. Blood 1992;11:2930-2936.
88. Rodgers GM, Conn MT. Homocysteine, an atherogenic stimulus, reduces protein C activation by arterial and venous endothelial cells. Blood 1990;75:895-901.
89. Franken DG, Vreugdenhil A, Boers GHJ, Verrips A, Blom HJ, Novakova IRO. Familial cerebrovascular accidents due to concomitant hyperhomocysteinemia and protein C deficiency type 1. Stroke 1993;24:1599-1600.
90. Mandel H, Brenner B, Berant M, Rosenberg N, Lanir N, Jakobs C, Fowler B, Seligsohn U. Coexistence of hereditary homocystinuria and factor V Leiden -

effect on thrombosis. N Eng J Med 1996;334:763-768.
91. Giannini MJ, Coleman M, Innerfield I. Antithrombin activity in homocystinuria. Lancet 1975;ii:1094.
92. Munnich A, Saudubray J-M, Dautzenberg M-D, Parvy P, Ogier H, Girot R, Manigne P, Frezal J. Diet-responsive proconvertin (Factor VII) deficiency in homocystinuria. J Ped 1983;102:730-734.
93. Palareti G, Salardi S, Piazzi S, Legnani C, Poggi M, Grauso F, Caniato A, Coccheri S, Cacciari E. Blood coagulation changes in homocystinuria: Effects of pyridoxine and other specific therapy. J Ped 1986;109:1001-1006.
94. Bienvenu T, Chadefaux B, Ankri A, Leblond V, Coude M, Salehian B, Binet J, Kamoun P. Antithrombin III activity is not related to plasma homocysteine concentrations. Hemostasis 1991;21:65-67.
95. Rodgers GM, Kane WH. Activation of endogenous factor V by homocysteine-induced vascular endothelial cell activator. J Clin Invest 1986;77:1909-1916.
96. Uhlemann ER, Tenpas JH, Lucky AW, Schulman JD, Mudd SH, Schulman NR. Platelet survival and morphology in homocystinuria due to cystathionine synthase deficiency. N Eng J Med 1976;295:1283-1286.
97. Hill-Zobel RL, Pyeritz RE, Scheffel U, Malpica O, Engin S, Camargo EE, Abbott M, Guilarte TR, Hill J, McIntyre PA, Murphy EA, Tsan M-F. Kinetics and distribution of $_{111}$Indium-labeled platelets in patients with homocystinuria. N Eng J Med 1982;307:781-786.

THREE DIFFERENT METHODS FOR THE DETERMINATION OF TOTAL HOMOCYSTEINE IN PLASMA.

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Introduction

Over the last decade mild to moderate hyperhomocysteinemia became an established risk factor for premature vascular disease (see for reviews[1,2]), and more recently for obstetric complications such as recurrent miscarriage, gross placental infarcts and neural tube defects[3]. In addition, the determination of total homocysteine in plasma is applied for the diagnosis and follow-up of folate and cobalamin deficiencies as well as the rare inborn errors of cobalamin metabolism, cystathionine synthase and methylenetetrahydrofolate reductase deficiency[1].

The increasing interest in the determination of total homocysteine in plasma has lead to the development of several different methods. This paper concerns three different methods for total homocysteine determination. One method is using a classic amino acid analyzer (AAA)[4] and the other two are high performance liquid chromatography (HPLC) methods[5,6].

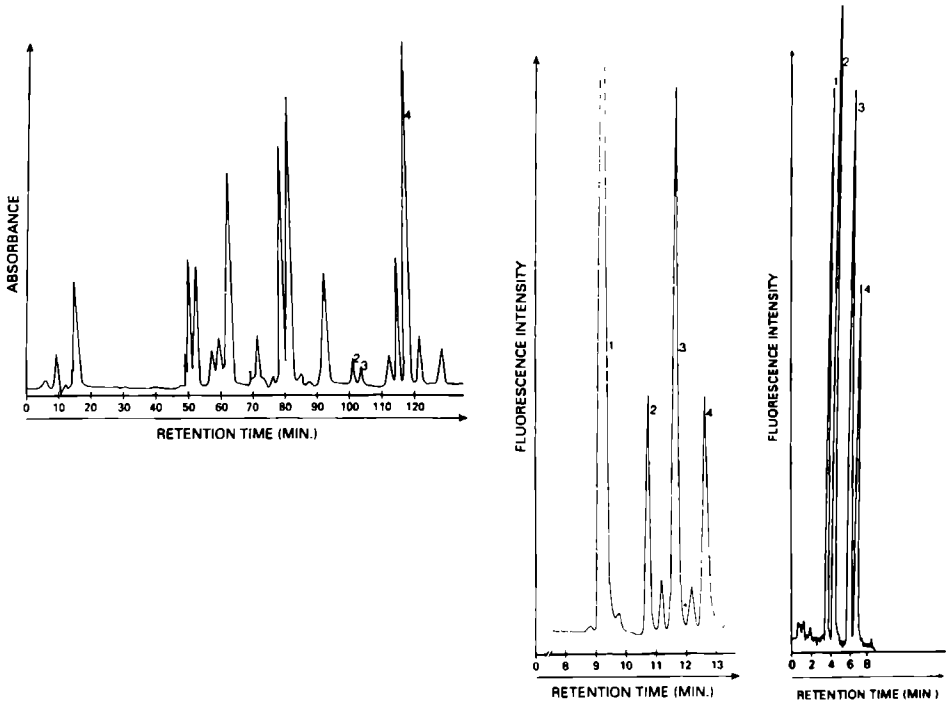

Figure 3.1. (a) Amino acid analyzer chromatogram of ninhydrin derivatives at 570 nm in pooled plasma. The total homocysteine concentration in plasma is spiked with DL-homocysteine to 40.0 μmol/L. Peaks: 1 = cysteine, 2 = homocysteine, 3 = methionine, 4 = norleucine. (b) HPLC chromatogram of mBrB derivatized thiols in a pooled plasma. The total homocysteine concentration is 40.0 μmol/L. Peaks: 1 = cysteine, 2 = cysteinylglycine, 3 = homocysteine, 4 = cysteamine. (c) HPLC chromatogram of SBD-F derivatized thiols in a pooled plasma. The total homocysteine concentration is 40.0 μmol/L. Peaks: 1 = cysteine, 2 = cysteamine, 3 = cysteinylglycine, 4 = homocysteine.

Materials and methods

The **AAA method** (Pharmacia/LKB, Alpha-Plus) applied dithiotreitol (DTT) for the reduction of the disulphide bounds and postcolumn derivatisation with ninhydrin, essentially according to Andersson et al.[4]. The column was equilibrated with 0.6 mol/L lithiumcitrate, pH 3.02 for 45 min. Next a buffer containing 1.0 mol/L lithiumcitrate, pH 3.45 eluted respectively homocysteine, methionine and norleucine (Figure 3.1a) from the ion exchange column (272 mm, I.D. 4.6 mm and 6 μm particle size) with a flow rate of 0.3 mL/min. To 0.15 mL of thawed plasma, 0.15 mL DTT (40 mM; pH 9.0) was added, mixed and incubated for 10 min at

room temperature. The mixture was deproteinized by adding 0.15 mL of a sulphosalicylic acid solution (25% w/v) containing L-norleucine (306 μmol/L) as an external standard. After vortexing, the mixture was kept on ice for 10 min and then centrifuged at 3500xg for 10 min. The supernatant was filtered (0.45 μm, Millipore) just before analysis. The absorption intensities of ninhydrin derivatives were measured at 440 and 570 nm.

The **HPLC $NaBH_4$/mBrB method** used $NaBH_4$ for reduction and mBrB for derivatization essentially according to Fiskerstrand et al.[5] with cysteamine as external standard. A programmable sample processor (Gilson model 232 BIO, Dilutor 401) was used for automated homocysteine reduction, derivatization and sample injection. After equilibration with 30 mmol/L ammoniumnitrate, 40 mmol/L ammoniumformate and 4 mmol/L tetrabutylammoniumhydrogensulphate (pH 3.2) the thiols derivatives were eluted from the column (Supelcosil LC-18 15 cm, 4.6 mm, 3 μm) with a 0 to 10.5% acetonitrile gradient in 11 min with a flow rate of 1 mL/min (Figure 3.1b).

HPLC TBP/SBD-F method applied TBP for reduction and SBD-F as derivatization agent according to Ubbink et al.[6] with some modifications. For derivatization 100 μL plasma and 50 μL of internal standard (cysteamine 0.125 mM) were mixed with 15 μL 10% TBP in methylformamide and incubated for 30 min at 4°C. The solution was deproteinized with 0.15 mL 10% trichloroacetic acid containing 1 mmol/L Na_4EDTA under vortexing, followed by centrifugation at 10 min at 4000xg. A 50 μL aliquot of the clear supernatant was mixed with 15 μL sodiumhydroxide (1.55 mol/L), 125 μL borate buffer (0.125 mol/L), pH 9.5 containing 4 mmol/L EDTA, and 50 μL SBD-F (1 mg/mL dissolved in borate buffer) and incubated for 60 min at 60°C. The sample was diluted with eluent (1:5) and 20 μL of the mixture was injected. The SBD-F derivatives were eluted isocratic from the column by 0.1 mM KH_2PO_4/5% acetonitril (pH 2.15) with a flow rate of 1 mL/min (Figure 3.1c).

EDTA plasma samples, not spiked, of controls and of vascular patients with severe or mild hyperhomocysteinemia were used for correlation studies.

Results and Discussion

The detection limits of all these three methods were low (less than 0.5 μmol/L) and only small amounts (less than 150 μL) of plasma were required with good reproducibility. The inter and intra variation coefficients were less than 6.5%.

A disadvantage of the amino acid analyzer method is the long injection to injection time of 138 min compared to 20 min or less for the HPLC methods. On the other hand the amino acid analyzer method measures also other amino acids involved in homocysteine metabolism such as methionine, cystathionine and cysteine. A disadvantage of both the amino acid analyzer and the HPLC TBP/SBD-F method is that they are not fully automated whereas the HPLC NaBH4/mBrB method is fully automated for unattended analysis of 105 samples.

The correlation between the HPLC NaBH4/mBrB method versus the AAA-method is $Y = 0.72 + 1.07X$, $R = 0.985, N = 80$. The total homocysteine concentrations (free and protein bound) in plasma of controls and patients varied from 4 to 156 μmol/L. The correlation between the HPLC NaBH4/mBrB method

versus the HPLC TBP/SBD-F method is $Y = 0.14 + 1.04X$ $R = 0.991$, $N = 37$, and the total homocysteine concentrations in plasma of controls and patients varied for 9 to 173 μmol/L.

The good correlation coefficients between the different methods show that despite the use of different kinds of reducing agents the same amount of total homocysteine is recovered from the plasma.

The HPLC NaBH4/mBrB method was modified and improved by using: 1. cysteamine as an external standard whereas Fiskerstrand et al.[5] used no standard; 2. addition of tetrabutylammoniumhydrogensulphate to the mobile phase for improvement of separation of the homocysteine peak from the fluorescent side products of mBrB; 3. reducing the flow rate from 1.5 mL/min to 1.0 mL/min; 4. increasing the total capacity for unattended analysis from 60 to 105 samples. Therefore this method is preferable for measurements of large amounts of samples. The HPLC TBP/SBD-F method[6] was modified by reducing volumes during derivatisation to economize the use of samples and reagents during derivatisation and the use of cysteamine as external standard.

In conclusion, all three studied methods are appropriate for the diagnosis of mild and severe hyperhomocysteinemia or cobalamin/folate deficiency in a routine laboratory setting.

References

1. Mudd SH, Levy HL, Skovby F. Disorders of transsulfuration. In: Scriver CR, Beaudet AL, Sly WS, Valle D (eds). The metabolic basis of inherited disease. McGraw-Hill Inc., New York, 1989;693-734.
2. Ueland PM, Refsum H, Brattstrom L. Plasma homocysteine and cardiovascular disease. In: Francis RB (ed). Atherosclerotic cardiovascular disease, hemostasis and endothelial function. Marcel Dekker Inc., New York, 1992;183-236.
3. Steegers-Theunissen RPM, Boers GHJ, Blom HJ, Trijbels JMF, Eskes TKAB, Hyperhomocysteinemia and recurrent spontaneous abortion or abruptio placentae. The Lancet 1992;339:1122-1123.
4. Andersson A, Brattstrom L. Isaksson A, Israelsson B, Hultberg B. Determination of homocysteine in plasma by ion-exchange chromatography. Scand J Clin Lab Invest 1989;49:445-450.
5. Fiskerstrand T, Refsum H, Kvalheim G, Lilletvedt B, Ueland PM. An improved automated method for the determination of homocysteine and other thiols in plasma and urine performance, practicalities and sample stability. Clin Chem 1993;39:263-271.
6. Ubbink JB, Vermaak WHJ, Bissbort S. Rapid high performance liquid chromatographic assay for total homocysteine levels in human serum. J Chromatogr 1991;565:441-446.

PART 1

ESTABLISHMENT OF INHERITED CAUSES OF HYPERHOMOCYSTEINEMIA

PREVALENCE OF FAMILIAL MILD HYPERHOMOCYSTEINEMIA

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Abstract

Previous studies have shown that elevated basal homocysteine levels are correlated among family members of patients with coronary vascular disease and juvenile venous thrombosis. This suggests the possibility of the presence of inherited basal mild hyperhomocysteinemia (mHH). We studied homocysteine levels, fasting as well as after methionine load, among 96 family members of 21 post-load hyperhomocysteinemic vascular index patients, i.e. 6 parents, 27 offspring, 38 siblings, 19 uncles and aunts, and 6 cousins. In 15 out of 21 screened families post-load mHH was established in at least one family member. Fasting and post-load mHH was observed in 19 out of 89 (21%) screened family members (fasting homocysteine levels not measured in seven family members), and 31 out of 96 screened family members (32%), respectively. In 40% of all family members, post-load mHH was not accompanied by fasting mHH. We conclude that both fasting and post-load mHH seems to be inherited in the majority of hyperhomocysteinemic vascular patients.

Introduction

Mild hyperhomocysteinemia (mHH), a risk factor for premature arteriosclerosis, is detected in a frequency of 9% to 47% in patients with premature cerebral, peripheral, or coronary stenotic arterial disease or with thromboembolism[1 11]. In the general population mHH is present in up to 8%[1 2]. MHH can be caused by enzymatic defects such as heterozygosity for cystathionine synthase (CS) deficiency, or homozygosity for thermolabile 5,10-methylenetetrahydrofolate reductase (MTHFR) deficiency, indicating a genetic cause in at least a subset of the detected patients[3 4 12 15]. Environmental influences such as vitamin B_{12} and folic acid deficiency, and renal or liver disease can also induce mHH[1 16 17].

Homocysteine blood concentrations were found to be correlated in monozygotic and dizygotic healthy twins[18 19] and were strongly correlated among family members of patients with coronary vascular disease[20-22]. These studies in family members of arterial occlusive diseased patients were performed on the base of fasting homocysteine concentration measurements only. Falcon et al.[8] reported high plasma homocysteine levels in 5 out of 8 families of patients with juvenile venous thrombosis.

In the present study, we have performed methionine loading tests in 96 family members of 21 hyperhomocysteinemic vascular patients. In these patients as well as their family members vitamin deficiencies or liver or renal failure which could lead to secondary mHH had been excluded. The prevalence of mHH, fasting as well as after methionine loading, among the family members was assessed to examine the possibility of the presence of an inherited cause of mHH in the vascular patients.

Materials and methods

Methionine Loading Test and Determination of Hyperhomocysteinemia

In 21 vascular disease patients hyperhomocysteinemia was established on the basis of their homocysteine level after methionine loading exceeding the mean + 2 standard deviations (SD) post-load homocysteine level in 95 controls. Methionine loading tests (0.1 g L-methionine/kg body weight) were performed in the family members according to a protocol reported previously[23]. Plasma samples in the fasting state and 6 hours after methionine load were collected and centrifuged instantly. The total homocysteine concentration (free plus protein bound) was measured by high-performance liquid chromatography[24]. At the time of screening, the index patient of family 10, his parents and sibships, and one cousin (fasting and post-load homocysteine levels of 11 and 46 μmol/L, respectively) presented with decreased vitamin B_{12} levels. Hypercholesterolemia was detected in the mother of family 4 (8.3 mmol/L; reference value $\leq$ 6.5 mmol/L), in the index patient of family 13 (7.7 mmol/L), hypertriglyceridemia was detected in the index patient of family 8 (5.46 mmol/L; reference value $\leq$ 2.00 mmol/L), and diabetes in the index patient of family 3 (fasting glucose: 9.4 mmol/L; reference value $\geq$ 7.8 mmol/L). All other index patients and family members had vitamin B_6, vitamin B_{12}, folic acid, glucose, cholesterol, triglycerides levels within the normal ranges, were without liver or renal disease, or hypertension of nonrenovascular origin. From all patients and the family members information of intake of vitamin supplements and medical history was obtained None of the 21 families and the studied parents

were related to one other within the second generation.

Index Vascular Patients

Of the 21 hyperhomocysteinemic index patients with documented vascular disease, 10 patients had suffered from cerebral occlusive arterial disease, 3 from peripheral occlusive arterial disease (2 intermittent claudication, and 1 subclavian arterial occlusion), 2 from anterior spinal arterial syndrome, 2 from coronary occlusive arterial disease, 3 from venous thrombosis (1 deep crural thrombosis and 2 cerebral thrombosis), and 1 from concomitant coronary occlusive arterial disease and deep crural thrombosis. One family (Figure 4.1, family number 13) was also known with inherited protein C deficiency[25]. The vascular disease became symptomatic in the index patients between their 16th and 50th year of age (38 ± 8 years; mean ± SD), the mean age ± SD at their screening was 39 ± 7 years.

	vascular index patient (n = 21)	FM (n = 96)	FM with elevated post-load (n = 31)	FM with normal post-load (n = 65)	Controls (n = 95)
fasting	25 ± 16 (n = 20)[4]	17 ± 14 (n = 89)[4]	27 ± 21 (n = 29)[4]	12 ± 3 (n = 60)[1]	11 ± 3
post-load	85 ± 28[4]	47 ± 20[4]	69 ± 20[4]	37 ± 8	37 ± 13
FA	9.3 ± 2.3 (n = 20)[4]	11.3 ± 3.4[4]	9.3 ± 2.0[4]	12.3 ± 3.5 (n = 64)[2]	14.4 ± 4.6
B_6	52 ± 16 (n = 20)	48 ± 14	44 ± 14[2]	50 ± 14 (n = 64)	52 ± 19
B_{12}	280 ± 136 (n = 20)	251 ± 85	238 ± 98	258 ± 77 (n = 64)	265 ± 101
age	38 ± 10	33 ± 12[3]	33 ± 14[1]	33 ± 12[3]	41 ± 9
sex (m/f)	4/17	42/54	17/14	25/40	23/72

Table4.1. Mean ± SD of laboratory characteristics of 21 vascular index patients with post-load mild hyperhomocysteinemia, 96 of their family members (FM), and 95 controls. The family members are divided in 31 family members with post-load mild hyperhomocysteinemia, and 65 family members with normal post-load homocysteine concentration. FA = plasma folic acid, B_6 = blood vitamin B_6, B_{12} = blood vitamin B_{12}. The age is given in years. M = male, F = female. [1] < 0.05, [2] < 0.01, [3] < 0.0001, [4] < 0.00001 (Wilcoxon Rank Sum W-test).

Family Members

The methionine loading tests in the studied family members were performed on their own request after detection of the index patient, except for 2 families (Figure 4.1, family number 10 and 13) which were asked to volunteer in the screening of mHH. The index patients of these 2 families were exceptionally young, i.e. 16 years of age at the time of their vascular incident. Only those families have been included in this study, in whom at least one complete family tree, i.e. both parents, or all children, or all brothers ánd sisters, had been screened for hyperhomocysteinemia. The 21 index patients had in total 131 first degree family members, i.e. 42 non-consanguineous parents, 30 offspring, and 59 siblings. Homocysteine concentrations, fasting and post-load, were determined in 72 out of these 131 family members (29 male and 43 female); i.e. 6 parents, 27 offspring, and 39 siblings. In family number 10 and 13 (Figure 4.1) 10 uncles and 9 aunts were also tested. These second degree family members were siblings of the fathers of both index cases in whom post-load mHH was revealed. In family number 10 another 6 third degree relatives (offspring of a post-load hyperhomocysteinemic second degree family member) were screened with the methionine loading test. At the time of screening the mean age ±SD of the 97 family members was 33 ± 9 years (range 12 to 68 years). One sister used vitamin supplements and was excluded from the study (Figure 4.1, family number 7). Four family members suffered from vascular complications at the time of screening (Figure 4.1, family number 4, 7, 13, 15). Their age at onset of vascular complications ranged from 37 to 59 years of age (mean ±SD 44 ± 8 year).

Control Subjects

Because of previously observed differences in mean fasting and post-load homocysteine levels between male, premenopausal and postmenopausal female control subjects the studied index patients and family members were categorized accordingly[3,26]. The fasting and post-load homocysteine concentration (2.5 - 97.5 percentile) respectively was in control men 8 - 18 and 25 - 54 μmol/L (n = 23), in control premenopausal women 6 - 15 and 18 - 51 μmol/L (n = 46), and in control postmenopausal women 6 - 19 and 25 - 69 μmol/L (n = 26)[27].

Results

Post-load mild hyperhomocysteinemia (Figure 4.2a, and Table 4.1)

In 15 out of the 21 screened families post-load mHH was established in at least one family member. In 31 out of 96 screened family members post-load mHH was observed (32%). Post-load mHH was detected in 50% of the parents (3 out of 6 parents), 26% of the offspring (7 out of 27 children), and in 29% of the siblings (11 out of 38 siblings among 16 index vascular patients). Seven out of 19 screened second degree and 3 out of 7 screened third degree family members revealed post-load mHH. By definition, the post-load homocysteine concentrations of vascular index patients, and post-load hyperhomocysteinemic family members were significantly higher than in controls (Table 4.1). Also the total group of studied family members showed significantly higher levels than controls. Plasma folic acid concentrations of vascular index patients, family members as one group and as subgroups with and without post-load hyperhomocysteinemia, were statistically significantly lower compared to controls. Plasma vitamin B_6 was

significantly lower in post-load hyperhomocysteinemic family members (Table 4.1). There was no evident sex difference among the hyperhomocysteinemic family members (17 of the 42 male, and 14 of the 54 female family members; chi-square $p > 0.05$).

Fasting mild hyperhomocysteinemia (Figure 4.2b, and Table 4.1)

Fasting mHH was present in 19 out of 89 family members (21%), including 17 among 29 post-load hyperhomocysteinemic family members, and 2 among 60 post-load normohomocysteinemic family members (fasting homocysteine levels not measured in 7 family members). Thus, in the included family members, in 40% post-load hyperhomocysteinemia was not accompanied by fasting elevated levels.

Fasting homocysteine levels were within the normal range in 6 out of 20 index patients (Figure 4.1, family number 3, 4, 17, 18, 19, 20; fasting homocysteine level not measured in 1 index patient). In 5 out of the 6 families of these 6 patients, one more post-load hyperhomocysteinemic family member was detected, and also in all these 5 family members the fasting homocysteine level was within the normal range. In the 14 families, in whom the index case showed elevated fasting homocysteine level, only 17 out of 24 post-load hyperhomocysteinemic family members showed also elevated fasting homocysteine levels (71%).

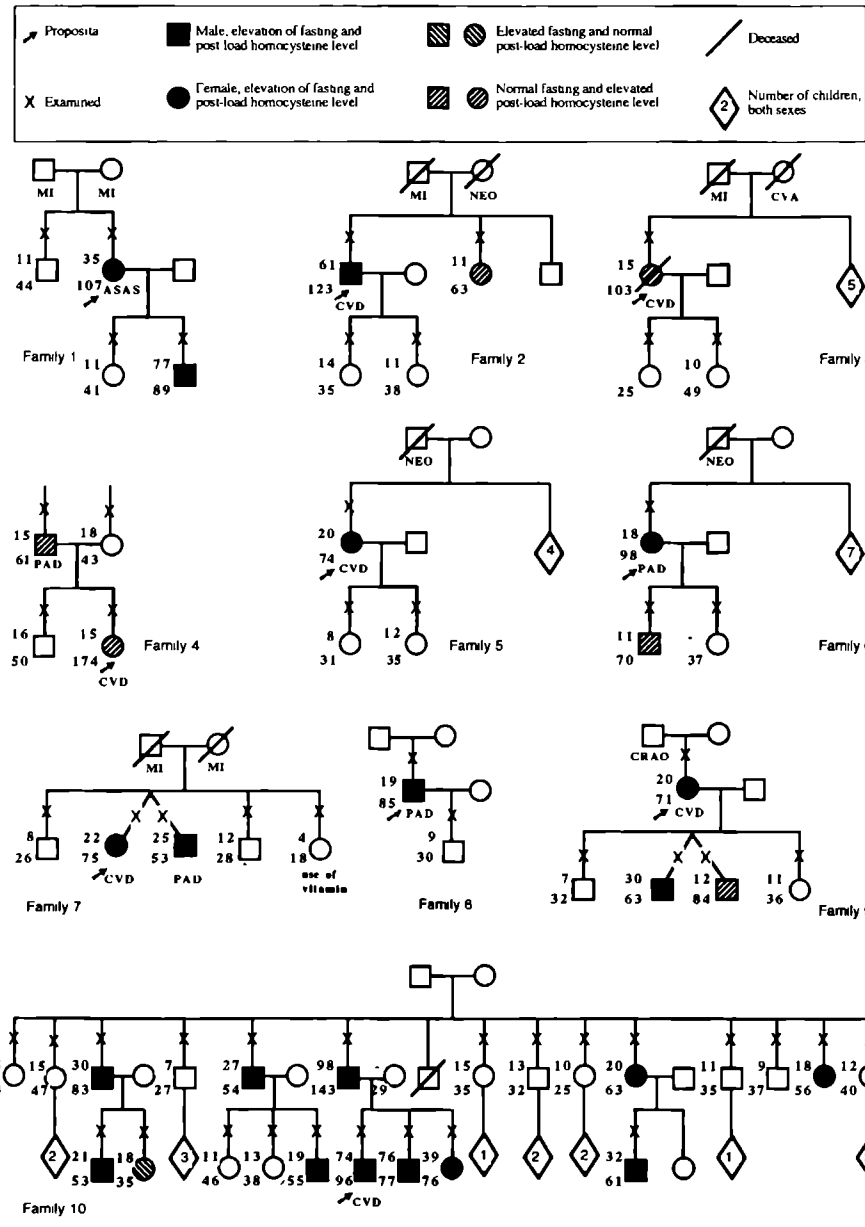
The fasting homocysteine levels were statistically significantly higher in vascular index patients, in family members as one group, in post-load hyperhomocysteinemic family members, but also, albeit less strongly significantly, in post-load normohomocysteinemic family members compared to the control group (Table 4.1).

Vascular complications in family members

Four screened family members were known with vascular manifestations at the time of the methionine loading test. Three of them revealed post-load mHH (Figure 4.1, family number 4, 7 and 13). The fourth vascular affected family member (Figure 4.1, family number 15) showed a high but normal post-load homocysteine concentration (fasting homocysteine level was not measured).

Discussion

In the present study, 71% of the families of a patient with various forms of arteriosclerosis had at least one other family member with post-load mHH. In 6 out of the 21 studied families no other relative had post-load mHH, but only 15 of the 40 first degree relatives were studied, and therefore, hyperhomocysteinemic family members could have been missed. Mildly elevated homocysteine levels were present in family members in 21% on the base of fasting and in 32% on the base of post-load homocysteine concentration. Our observations are in line with previous reports in which basal homocysteine concentration in healthy twins and in families of coronary vascular patients is reported to show a significant correlation[18-22]. The present study demonstrates that both fasting and post-load mHH, in the absence of vitamin deficiencies and renal or liver insufficiencies, seems to be inherited in the majority of vascular patients. Further enzymatic and molecular studies are needed to reveal the involved genetic enzymatic defects and their inheritance patterns. Reduced activities in the range of obligate carriers for CS-deficiency have been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Normal fasting and elevated post-load homocysteine level
2 Number of children, both sexes
MI
NEO
CVA
ASAS
CVD
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Family 2
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Family 5
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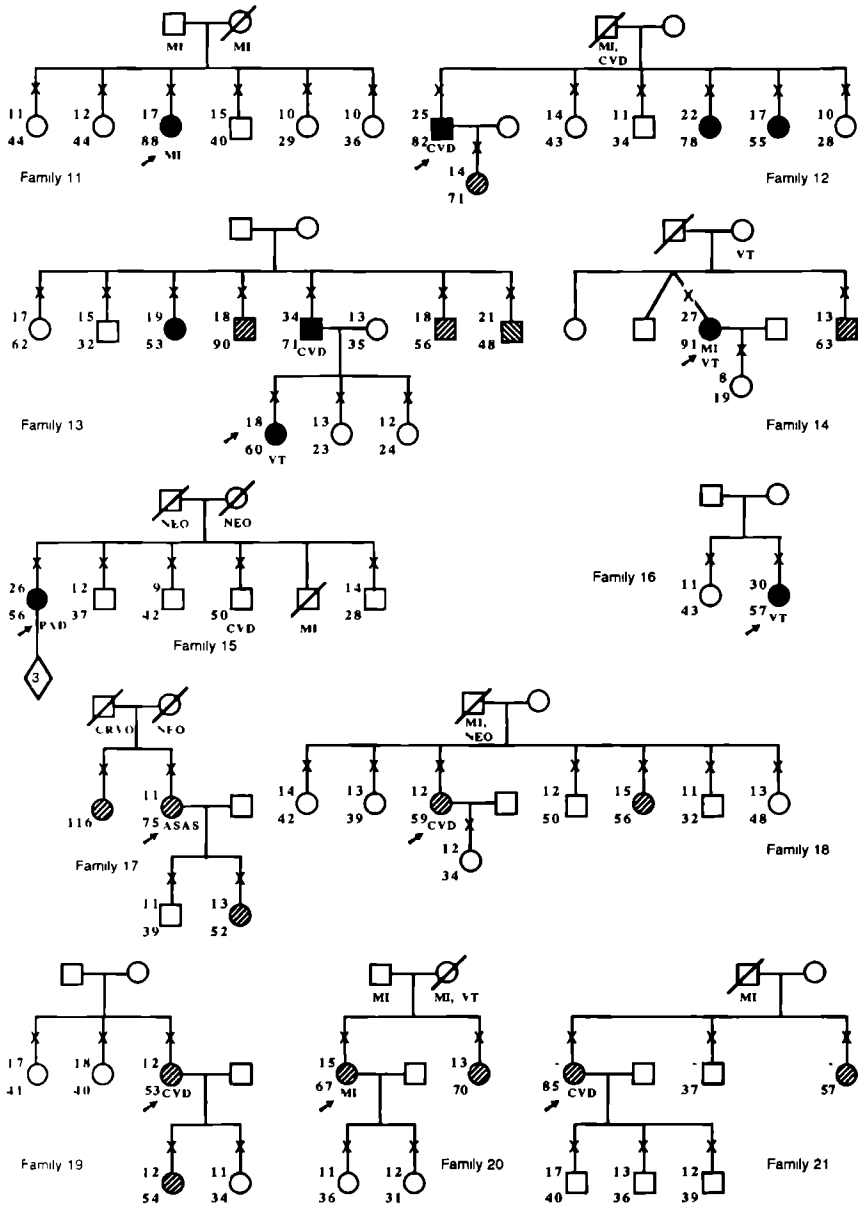

Figure 4.1. Twenty-one family pedigrees of vascular patients with post-load mild hyperhomocysteinemia. At the left side of the symbols, the total homocysteine concentration before (above) and after methionine loading (below) are shown. CVD indicates cerebral vascular disease; PAD, peripheral arterial disease; MI, myocardial infarction; VT, venous thrombosis; ASAS, anterior spinal artery syndrome; CRAO, central retinal artery occlusion; CRVO, central retinal venous occlusion; and Neo, neoplasm.

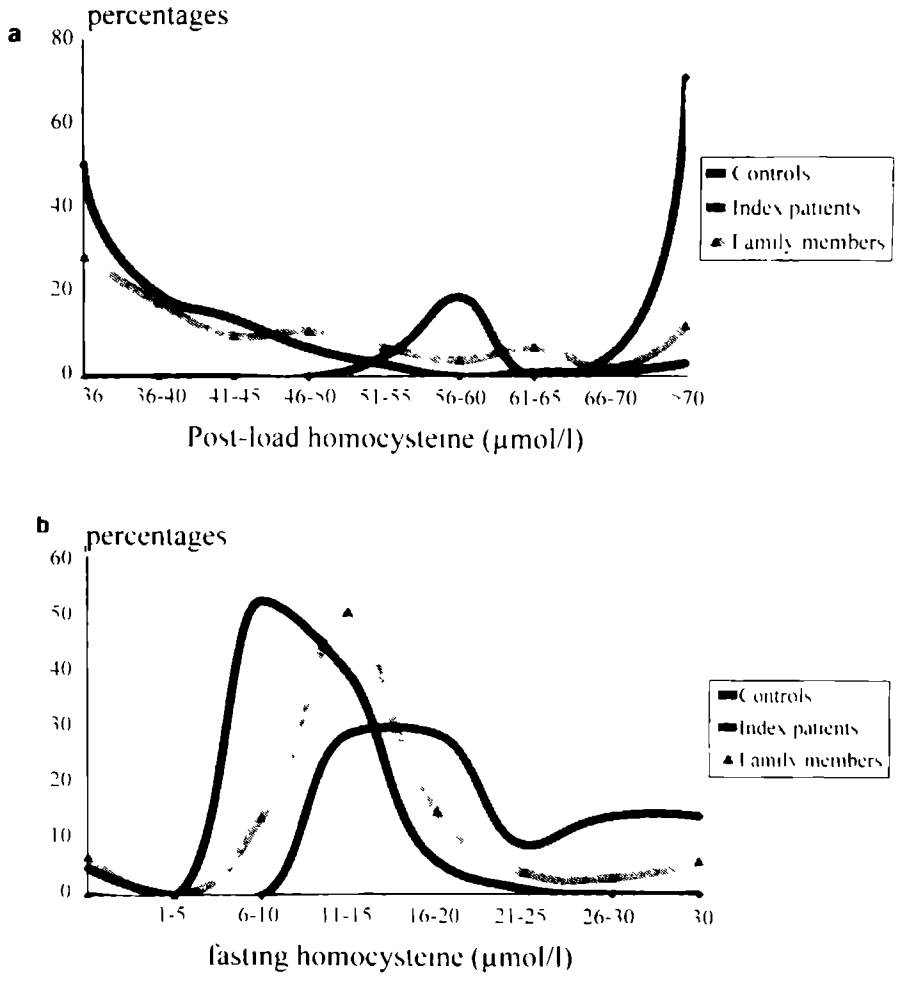

Figure 4.2a and 4.2b. Frequency distribution in percentages of post-load (Figure 4.2a) and fasting (Figure 4.2b) plasma homocysteine concentrations in controls (n = 95), index patients (n = 21), and family members of the index patients (n = 96). Post-load homocysteine concentrations above 54 µmol/L in men, 51 µmol/L in premenopausal women, and 69 µmol/L in postmenopausal women are defined as hyperhomocysteinemia.

reported in hyperhomocysteinemic vascular patients[3,4]. This finding was difficult to reconcile with the conclusion by Mudd et al.[28] of a normal incidence of heart attacks or strokes in a large group of obligate heterozygotes for CS-deficiency studied by questionnaires. Furthermore, even in populations with the highest prevalence of homozygous CS-deficiency, such as in Ireland, the calculated number of carriers is too low to account for the number of observed hyperhomocysteinemic vascular patients[29]. Indeed, very recently, it was reported that the finding of lowered CS-activity was not reproducible in 96% of studied hyperhomocysteinemic vascular patients[15,30]. Moreover, in the Netherlands where the 833 T → C transition is detectable as the mutation in the CS gene on chromosome 21 in 50% of alleles of homozygotes for CS-deficiency, in 60 cardiovascular patients this mutation could not be identified in any of them[30]. The same holds true for the observation in Ireland where the 919 G → A mutation in the CS gene, determined in 70% of Irish homocystinuric alleles, could not be detected in a group of 100 Irish patients with premature vascular disease[31]. From all these enzymatic and molecular genetic studies summarizing, it can be concluded that there is no evidence, so far, that heterozygosity for CS-deficiency plays a role as the basis of mHH in vascular patients. In a previous study, we observed an incidence of a thermolabile MTHFR enzyme in 28% of hyperhomocysteinemic cardiovascular patients[15]. Such thermolability is consistently associated with the 677 C → T mutation in the MTHFR gene on chromosome 1[12], and a subject has to be homozygous for such mutation to produce hyperhomocysteinemia[13,14]. Despite a high incidence of this thermolabile MTHFR enzyme among hyperhomocysteinemic patients and controls, i.e. 28% and 5%, respectively[30], the frequency of this defect does not seem sufficient enough to provide in its own the base of all familial occurrence of mHH as observed in the present study.

It is still questionable whether either fasting or post-load homocysteine concentration is the most sensitive indicator of abnormal homocysteine metabolism. Both fasting and post-load mHH are considered risk factors for cardiovascular disease[1,6,32]. In case we screened for mHH only on the base of fasting homocysteine levels, no more than 14 out of 20 index patients (70%) and 17 out of 29 family members with post-load mHH (59%) would have been considered hyperhomocysteinemic. Even if family members of only the 14 index patients with fasting mHH had been screened for fasting homocysteine levels alone, 29% (7 out of 24) of the family members who indeed showed post-load mHH, would have been classified as normohomocysteinemic. Post-load elevated homocysteine levels in the absence of fasting hyperhomocysteinemia is probably on the basis of another enzymatic defect than concomitant fasting and post-load hyperhomocysteinemia[33]. In our present study, we have screened family members of vascular index patients with post-load elevated homocysteine levels. None of the 21 index patients were known with fasting elevated and post-load normal homocysteine concentrations. Therefore, a selection in families has been made on the basis of this inclusion criterion. However, studies on fasting homocysteine levels in healthy twins and also in families of cardiovascular patients have been performed already previously, showing a strong correlation[18-22]. We prefer to perform both fasting and post-load homocysteine concentrations in vascular patients and family members to investigate their individual homocysteine status more completely[11,25,34-37].

Plasma folic acid concentrations, were significantly lower in vascular index patients, in family members as one group, and in the subgroups of post-load normo- and hyperhomocysteinemic family members, compared to controls. This finding is in accordance with earlier observations of reduced though not deficient folic acid blood levels in cardiovascular patients[4,10,11]. Establishing both in normo- and hyperhomocysteinemic family members lower folic acid compared to controls, could be an indication of a common habit of low folate intake in these families, leading to hyperhomocysteinemia in some of the members. However, in family members with hyperhomocysteinemia, the decrease in folic acid concentration was much more pronounced than in their normohomocysteinemic family members, comparable with the highly significantly lower levels in the vascular patients in these families. This is much more suggestive of the presence of a hereditary defect in homocysteine metabolism leading to a higher demand for folate in the hyperhomocysteinemic patients and their hyperhomocysteinemic family members.

Among the 96 investigated family members, 4 had known vascular disease. Three of them were hyperhomocysteinemic, and one had high-normal post-load homocysteine concentration. Therefore, screening for mHH of family members suffering from vascular events among families of a hyperhomocysteinemic patient, is recommended. About 90% of the family members with post-load mHH revealed no subjective signs of vascular disease, at least so far. Most screened family members were younger than their respective index case. This suggests that familial mild hyperhomocysteinemia leading to symptomatic vascular disease has a low expression, may be age dependent and that homocysteine in mild excess may require more triggering factors.

MHH in vascular patients can be normalized by vitamin B_6 and/or folic acid treatment[11,25,34-37]. In case homocysteine-lowering intervention in hyperhomocysteinemic vascular patients and their hyperhomocysteinemic family members is clinically beneficial in terms of preventing recurrent and first occurrence of arterial disease, respectively, such treatment will be of significant importance in general health care.

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References

1. Ueland PM, Refsum H, Brattström L. Plasma homocysteine and cardiovascular disease. In: Francis RBF. Atherosclerotic cardiovascular disease, hemostasis, and endothelial function. Marcel Dekker Inc., New York, 1992;183-236.
2. Kang S-S, Wong PWK, Malinow MR. Hyperhomocysteinemia as a risk factor for occlusive vascular disease. Annu Rev Nutr 1992;12:279-298.
3. Boers GHJ, Smals AGH, Trijbels JMF, Fowler B, Bakkeren JAJM, Schoonderwaldt HC, Kleijer WJ, Kloppenborg PWC. Heterozygosity for homocystinuria in premature peripheral and cerebral occlusive arterial disease. N Engl J Med 1985;313:709-715.
4. Clarke R, Daly L, Robinson K, Naughten E, Cahalane S, Fowler B, Graham I. Hyperhomocysteinemia: an independent risk factor for vascular disease. N Engl J Med 1991;324:1149-1155.
5. Brattström L, Lindgren A. Hyperhomocysteinemia as a risk factor for stroke. Neurol Res 1992;14:81-84.
6. Brattström L, Tengborn L, Lagerstedt C, Israelsson B, Hultberg B. Plasma homocysteine in venous thromboembolism. Haemostasis. 1991;21:51-57.
7. Bienvenu T, Ankri A, Chadefaux B, Kamoun P. Dosage de L'homocystéine plasmatique dans l'exploration des thromboses du sujet jeune. Presse Méd 1991;20:985-988.
8. Falcon CR, Cattaneo M, Panzeri D, Martinelli I, Mannucci PM. High prevalence of hyperhomocyst(e)inemia in patients with juvenile venous thrombosis. Arterioscler Thromb 1994;14:1080-1083.
9. den Heijer M, Blom HJ, Gerrits WBJ, Rosendaal FR, Haak HL, Wijermans PW, Bos GMJ. Is hyperhomocysteinemia a risk factor for recurrent venous thrombosis? Lancet 1995;345:882-885.
10. Israelsson B, Brattström LE, Hultberg BL. Homocysteine and myocardial infarction. Atheroscler 1988;71:227-233.
11. Brattström L, Israelsson B, Norrving B, Bergqvist D, Thörne J, Hultberg B, Hamfelt A. Impaired homocysteine metabolism in early-onset cerebral and peripheral occlusive arterial disease. Effects of pyridoxine and folic acid treatment. Atheroscler 1990;81:51-60.
12. Frosst P, Blom HJ, Milos R, Goyette P, Sheppard CA, Matthews RG, Boers GHJ, den Heijer M, Kluijtmans LAJ, van den Heuvel LP, Rozen R. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. Nat Genet 1995;10:111-113.
13. Kang SS, Wong PWK, Susmano A, Sora J, Norusis M, Ruggie N. Thermolabile methylenetetrahydrofolate reductase: an inherited risk factor for coronary artery disease. Am J Hum Genet 1991;48:536-545.
14. Kang SS, Wong PWK, Bock HGO, Horwitz A, Grix A. Intermediate hyperhomocyteinemia resulting from compound heterozygosity of methylenetetrahydrofolate reductase mutations. Am J Hum Genet 1991;48:546-551.
15. Engbersen AMT, Blom HJ, Franken DG, Boers GHJ, Stevens EMB, Poirot CM, Trijbels JMF. Thermolabile 5,10-methylenetetrahydrofolate reductase as a cause of mild hyperhomocysteinemia. Am J Hum Genet 1995;56:142-150.
16. Ueland PM, Refsum H. Plasma homocysteine, a risk factor for vascular disease: plasma levels in health, disease, and drug therapy. J Lab Clin Med 1989;473-501.
17. Ueland PM, Refsum H, Stabler SP, Malinow MR, Andersson A, Allen RH. Total homocysteine in plasma or serum: methods and clinical applications. Clin Chem 1993;39:1764-1779.
18. Reed T, Malinow MR, Christian JC, Upson B. Estimates of heritability of plasma homocyst(e)ine levels in aging adult male twins. Clin Genet 1991;39:425-428.
19. Berg K, Malinow MR, Kierulf P, Upson B. Population variation and genetics of plasma homocyst(e)ine level. Clin Genet 1992;41:315-321.

20. Genest JJ, McNamara JR, Upson B, Salem DN, Ordovas JM, Schaefer EJ, Malinow MR. Prevalence of familial hyperhomocyst(e)inemia in men with premature coronary artery disease. Arterioscler Thromb 1991;11:1129-1136.
21. Williams RR, Malinow MR, Hunt SC, Upson B, Wu LL, Hopkins PN, Stults BN, Kuida H. Hyperhomocyst(e)inemia in Utah siblings with early coronary disease. Coron Art Dis 1990;1:681-685.
22. Wu LL, Wu J, Hunt SC, James BC, Vincent GM, Williams RR, and Hopkins PN. Plasma homocyst(e)ine as a risk factor for early familial coronary artery disease. Clin Chem 1994;40:552-561.
23. Boers GHJ, Fowler B, Smals AGH, Trijbels JMF, Leermakers AI, Kleijer WJ, Kloppenborg PWC. Improved identification of heterozygotes for homocystinuria due to cystathionine synthase deficiency by the combination of methionine loading and enzyme determination in cultured fibroblasts. Hum Genet 1985;69:164-169.
24. te Poele-Pothoff MTWB,, van den Berg M, Franken DG, Boers GHJ, Jakobs C, de Kroon IFI, Eskes TKAB, Trijbels JMF, Blom HJ. Three different methods for the determination of total homocysteine in plasma. Ann of Clin Biochem 1995;32:218-220.
25. Franken DG, Vreugdenhil A, Boers GHJ, Verrips A, Blom HJ, Novakova IRO. Familial cerebrovascular accidents due to concomitant hyperhomocysteinemia and protein C deficiency type 1. Stroke 1993;24:1599-1600.
26. Boers GHJ, Smals AGH, Trijbels JMF, Leermakers AI, Kloppenborg PWC. Unique efficiency of methionine metabolism in premenopausal women may protect against vascular disease in the reproductive years. J Clin Invest 1983;72:1971-1976.
27. van den Berg M, Franken DG, Boers GHJ, Blom HJ, Jakobs C, Rauwerda JA. Combined vitamin B_6 plus folic acid therapy in young patients with arteriosclerosis and hyperhomocysteinemia. J Vasc Surg 1994;20:933-940.
28. Mudd SH, Havlik R, Levy HL, McRusick VA, Feinleib M. A study of cardiovascular risk in heterozygotes for homocystinuria. Am J Hum Genet 1981;33:883-893.
29. Daly L, Robinson K, Tan KS, Graham I. Hyperhomocysteinemia: a metabolic risk factor for coronary heart disease determined by both genetic and enviromental influences? J Med 1993;86:685-689.
30. Kluijtmans LA, van den Heuvel LPWJ, Boers GHJ, Frosst P, Stevens E, van Oost BA, den Heijer M, Trijbels JMF, Rosen R, Blom HJ. Molecular genetic analysis in mild hyperhomocysteinemia: a common mutation in the methylenetetrahydrofolate reductase gene is a genetic risk factor for cardiovascular disease. Am J Hum Genet 1996;58:35-41.
31. Whitehead AS, Ward P, Tan S, Naughten E, Kraus JP, Sellar GC, McConell DJ, et al. The molecular genetics of homocystinuria, hyperhomocysteinemia, and premature vascular disease in Ireland. In: Mato JM, Caballero A (eds). Methionine metabolism: molecular mechanisms and clinical implications. Bouncopy Inc., Madrid CSIC, 1994;79-83.
32. Stampfer MJ, Malinow MR, Willett WC, Newcomer LM, Upson B, Ullman D. A prospective study of plasma homocyst(e)ine and risk of myocardial infarction in US physicians. JAMA 1992;268:877-881.
33. Bostom AG, Jacques PF, Nadeau MR, Williams RR, Ellison RC, and Selhub J. Post-methionine load hyperhomocysteinemia in persons with normal fasting total plasma homocysteine: initial results from The NHLBI Family Heart Study. Atheroscler 1995;116:147-151.
34. Brattström LE, Israelsson B, Jeppsson J-O, Hultberg BL. Folic acid - an innocuous means to reduce plasma homocysteine. Scand J Clin Lab Invest 1988;48:215-221.
35. Dudman NPB, Wilcken DEL, Wang J, Lynch JF, Macey D, Lundberg P. Disordered methionine/homocysteine metabolism in premature vascular disease. Arterioscler Thromb 1993;13:1253-1260.

36. Franken DG, Boers GHJ, Blom HJ, Trijbels JMF, Kloppenborg PWC. Treatment of mild hyperhomocysteinemia in vascular disease patients. Arterioscler Thromb 1994;14;465-470.
37. Franken DG, Boers GHJ, Blom HJ, Trijbels JMF. Effect of various regimens of vitamin B_6 and folic acid treatment on mild hyperhomocysteinemia in vascular patients. J Inher Metab Disease 1994;17:159-162.

THERMOLABILE 5,10-METHYLENE-TETRAHYDROFOLATE REDUCTASE AS A CAUSE OF MILD HYPERHOMOCYSTEINEMIA

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Abstract

Thermolability of 5,10-methylenetetrahydrofolate reductase (MTHFR) was examined as a possible cause of mild hyperhomocysteinemia in patients with premature vascular disease. Control subjects, vascular patients with mild hyperhomocysteinemia and with normohomocysteinemia were studied. The mean (± SD) specific MTHFR activity in lymphocytes of 22 control subjects was 15.6 (± 4.7) nmol CH_2O/mg protein/h (range· 9.1 - 26.6) and the residual activity (± SD) after heat inactivation for 5 min at 46 °C was 55.3 (± 12.0)% (range: 35.9 - 78.3) By measurement of MTHFR activity, two distinct subgroups of hyperhomocysteinemic patients became evident. One group (n = 11) had thermolabile MTHFR with a mean (± SD) specific activity of 8.7 (± 2.1) nmol CH_2O/mg protein/h (range: 5.5 - 12.7) and a residual activity after heat inactivation ranging from 0 to 33%. The other group (n = 28) had normal specific activity (± SD) of 21 5 (± 7.2) nmol CH_2O/mg protein/h (range: 10.0 - 39.0) and a normal residual activity (± SD) of 53.8 (± 9.2)% (range: 33.1 - 71.5) after heat inactivation The mean (± SD) specific activity of 29 normohomocysteinemic patients was 20.7 (± 6.5) nmol CH_2O/mg protein/h (range: 9.4 - 33 8) and the mean (± SD) residual activity after heat inactivation was 58.2 (± 10.2)% (range: 43.0 - 82.0). Thus, in 28% of the hyperhomocysteinemic patients with premature vascular disease, abnormal homocysteine metabolism could be attributed to thermolabile MTHFR.

Introduction

Over the last decade, mild hyperhomocysteinemia has become an established risk factor for premature vascular disease[1 3] Homocysteine is presumed to damage the endothelial cells, although, the mechanism of its toxicity remains obscure[4].

Homocysteine accumulation may be caused by a metabolic block in either the degradation of homocysteine to cystathionine or in the remethylation of homocysteine to methionine (Figure 5.1). The classic form of severe hyperhomocysteinemia is caused by cystathionine ß-synthase deficiency. This enzyme catalyzes the formation of cystathionine from homocysteine and serine. Another enzymatic cause of severe hyperhomocysteinemia is 5,10-methylenetetrahydrofolate reductase (MTHFR) deficiency, which affects the remethylation of homocysteine to methionine. MTHFR catalyzes the reduction of 5,10-methylenetetrahydrofolate (MTHF) to 5-methyltetrahydrofolate (methyl-THF), in which flavin adenine dinucleotide (FAD) serves as cofactor (Figure 5.1).

Severe hyperhomocysteinemia due to MTHFR deficiency causes neurological abnormalities, mental retardation, arteriosclerosis and thrombosis[5]. The biochemical characteristics are hyperhomocystinuria, reduced or low methionine concentrations in plasma, low plasma folate levels and very low MTHFR activity in both fibroblasts and lymphocytes[6 8]. Low plasma folate levels result from a lack of the MTHFR product methyl-THF, which is the main form of circulating folate. The clinical severity and the extent of biochemical derangement appears to be correlated with the degree of enzyme deficiency[5 7]. In obligate heterozygotes for severe MTHFR deficiency the specific MTHFR activity is about 50% of the normal mean and it is unknown whether this condition is associated with an increased risk for vascular disease[9]

Heterozygotes for cystathionine ß-synthase deficiency have reduced enzyme activity in cultured fibroblasts and also mild hyperhomocysteinemia after methionine loading[10]. Reduced cystathionine ß-synthase activity in fibroblasts has also been found in the majority of vascular disease patients with mild hyperhomocysteinemia in two studies[11 12] However, this finding could not be reconciled with the observations of Mudd et al.[13] that in a large series of families with a homozygote cystathionine ß-synthase deficient patient the obligate carriers did not have an increased risk for vascular disease. While there is evidence of a high prevalence of mild hyperhomocysteinemia in patients with vascular disease[1 3] even in populations with the highest prevalence of homozygous cystathionine ß-synthase deficiency, such as in Ireland, the calculated number of heterozygotes is to low to account for the number of observed hyperhomocysteinemic vascular patients[14] In agreement with this observation, we (Blom, Fowler, Boers and Trijbels, unpublished observations) and others (Kraus, personal communication) could not reproduce the finding of lowered cystathionine ß-synthase activity in fibroblasts of vascular patients with mild hyperhomocysteinemia. This suggest that some aberration other than heterozygosity for cystathionine ß-synthase deficiency is causing the hyperhomocysteinemia in vascular patients.

Recently, a thermolabile variant of MTHFR[9 15 17] was shown to be caused by a mutation different from that causing the severe form of MTHFR deficiency. Homozygotes for thermolabile MTHFR deficiency have a specific activity of approximately 50% of normal and a residual activity after heat inactivation of less than 30%, compared to 50% residual activity in control subjects. Kang et al [9]

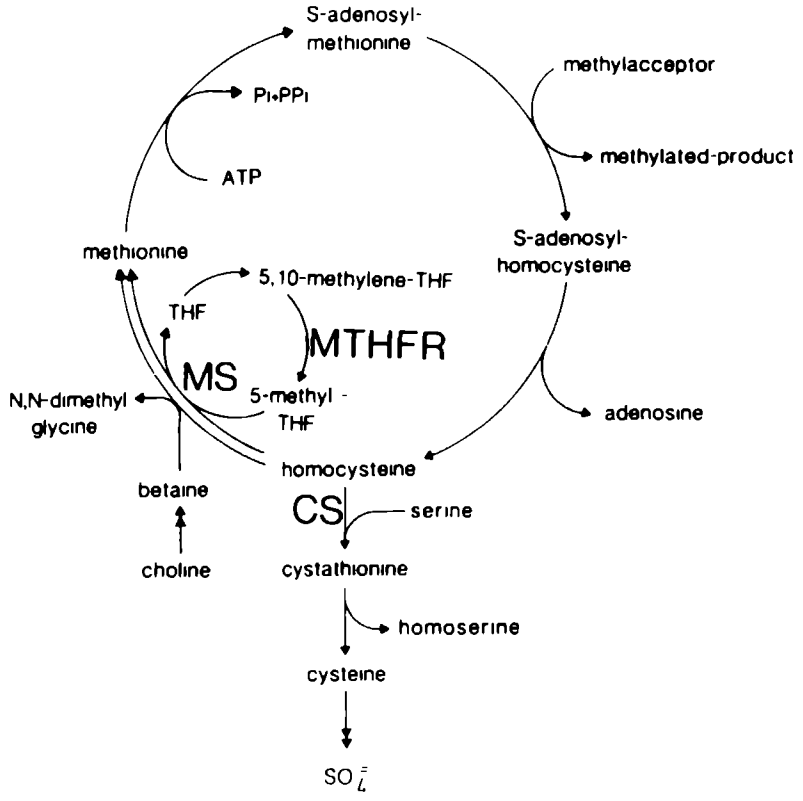

Figure 5.1. Homocysteine metabolism. CS: cystathionine ß-synthase; MTHFR: 5,10-methylenetetrahydrofolate reductase; MS: methionine synthase; THF: tetrahydrofolate.

reported an incidence of homozygote thermolabile MTHFR deficiency of 17% in a group of 212 patients with coronary artery disease. Obligate heterozygotes for thermolabile MTHFR deficiency have a specific enzyme activity of about 75% of the normal mean, but probably have thermostable MTHFR[17]. Compound heterozygotes for MTHFR deficiency with one allele for the severe form and one allele for the thermolabile form have also been described. These patients have a specific enzyme activity of about 25% of the normal mean and their MTHFR is probably thermolabile[17].

The present study describes a modified method for measurement of thermolabile MTHFR in isolated lymphocytes. Reference values were obtained from 23 healthy subjects. From our patient group with premature vascular disease[18] we selected 39 patients with and 29 without mild hyperhomocysteinemia to study the relationship between mild hyperhomocysteinemia and thermolabile MTHFR.

Patients and controls

Patients with arteriosclerosis or venous thrombosis under 55 years of age were screened for mild hyperhomocysteinemia by the methionine loading test (0.1

g L-methionine/kg body weight) in the Univeristy Hospital Nijmegen[11]. Patients with hyperlipoproteinemia, hypertension and diabetes mellitus were excluded. There were no other selection criteria.

Reference values for homocysteine concentrations, before and after methionine loading, were obtained from 88 control subjects, 65 premenopausal women and 23 men. Reference values for folic acid and vitamin B_{12} levels were obtained from 68 control subjects, 45 premenopausal women and 23 men. Mild hyperhomocysteinemia was defined as total homocysteine blood levels after methionine loading higher than the mean + 2SD of the reference group. The mean ± 2SD range of the reference group for total homocysteine concentration was 4 - 16 μmol/L (skewness: 0.9, kurtosis: 1.0) after fasting and 15 - 49 μmol/L after methionine loading (skewness: 0.7, kurtosis: -0.3). Mean ± 2SD range for plasma folic acid was 5.3 - 22.5 nmol/L (skewness: 1.1, kurtosis: 1.1) and for vitamin B_{12} 84 - 487 pmol/L (skewness: 0.9, kurtosis: 1.0). Four patients (number 8, 11, 16 and 23) were folate deficient (< 5.3 nmol/L) and one patient (number 14) had reduced vitamin B_{12} (79 pmol/L). However, these patients showed no classical clinical symptoms of folate or vitamin B_{12} deficiency.

As a reference, MTHFR activity was measured in a group of 23 healthy subjects consisting of hospital personnel without any clinical evidence for vascular disease. Their mean (± SD) age was 32.7 (± 7.4) years. One subject clearly showed thermolabile MTHFR and was excluded from the control group. The presence of thermolabile MTHFR was studied in several groups of vascular patients after their homocysteine status was established by methionine loading. One group with mild hyperhomocysteinemia consisted of 39 patients, 28 premenopausal women and 11 men, with proven vascular disease (17 patients with cerebral, 9 patients with peripheral and 5 patients with coronary arterial occlusive disease and 8 patients with venous thrombosis). The mean (± SD) age of these patients at the time of study was 40.3 (± 8.9) years. The mean (± SD) age of clinical onset of the vascular abnormalities in this group was 35.6 (± 10.1) years.

Another group consisted of 29 normohomocysteinemic patients, 14 female and 15 male, with premature vascular disease (21 patients with cerebral, 6 patients with peripheral and one patient with coronary arterial occlusive disease and one patient with venous thrombosis). Their mean (± SD) age at the time of study was 43.8 (± 12.5) years. The mean (± SD) age of clinical onset of vascular disease in this group was 40.3 (± 11.4) years.

Materials and methods

Potassium phosphate, L-(+)-ascorbic acid, 35% formaldehyde, toluene and acetic acid were obtained from Merck (Darmstadt, Germany). FAD (disodium salt), menadione sodium bisulfite, dimedone and methyl-THF (barium salt) were obtained from Sigma Chemical Co (St.Louis, USA). Ethylenediamine-tetraacetate (EDTA) was obtained from Fluka BioChemika (Buchs, Switzerland) and [Me-^{14}C] methyl-THF (50 mCi/mmol, barium salt) was obtained from Amersham International plc (Amersham, UK). An Eppendorf 5436 thermomixer (Hamburg, Germany) was used for preincubating the enzyme extract and for incubating the incubation mixture.

Lymphocytes were isolated from 20 ml heparinized blood using Lymphoprep (Nycomed Pharma AS, Oslo, Norway)[19] and were washed twice with Hank's buffer (ICN Biomedicals Inc, Costa Mesa, UK). The cell pellet was stored at - 80°C until

enzyme assay.

The MTHFR activity was determined radiochemically in lymphocytes, in its physiological reverse direction (Figure 5.1). [Me-^{14}C] Methyl-THF served as the substrate in the presence of menadione as electron acceptor. Activities were measured using a modified method of Kang et al.[9]. The cells were resuspended in 50 mM potassium phosphate buffer pH 7.2, frozen and thawed three times, and centrifuged 40 min at 15.8 x 10^3 g. A part of the supernatant was preincubated for 5 min at 46°C to determine the heat stability. FAD was omitted during this heat inactivation[20]. The incubation mixture, with a final volume of 600 µl, consisted of 0.18 M potassium phosphate buffer pH 6.8, 1.15 mM EDTA pH 7.0, 11.5 mM ascorbic acid, 54 µM FAD, 20 µM [Me-^{14}C] methyl-THF (5.0 x 10^5 dpm), 3.5 mM menadione and a maximum of 250 µl enzyme extract (preincubated supernatant or normal supernatant). The incubation was started by addition of menadione and lasted for 20 min in the dark at 37°C. The blank contained all the components of the incubation mixture, except enzyme extract. The incubation was terminated by the addition of 10 µl of 1.0 M carrier formaldehyde, 50 µmoles dimedone in 200 µl ethanol:water (1:1) and 100 µl 3.0 M potassium acetate, pH 4.5. The reaction mixture was heated at 95°C for 15 min, after which it was cooled on ice for about 10 min. The reaction mixture was added to 3.0 ml toluene and stirred vigorously for 15 sec. After low speed centrifugation, 2.0 ml of the toluene phase was taken for measurement of radioactivity. Protein was determined by the method of[21]. Enzyme activity is expressed as nmoles of formaldehyde formed/mg protein/h.

Cystathionine ß-synthase activity was measured as described elsewhere[10,22] without addition of pyridoxal phosphate to the assay-mixture in cultured fibroblasts of eight vascular hyperhomocysteinemic patients. For comparison cystathionine ß-synthase activities were also measured in fibroblasts of 13 obligate heterozygotes for cystathionine ß-synthase and 12 control subjects.

Total (free plus protein bound) homocysteine concentrations, fasting and after methionine loading, were measured in EDTA plasma by means of high performance liquid chromatography using fluorescence detection[23]. Folic acid and vitamin B_{12} levels were determined in heparinized plasma and vitamin B_6 levels in whole blood by routine hospital assays.

Statistics

Rank-sum-two sample test and the Fisher's exact test (2-tail) were applied. Normal distribution was supposed to be present in case the skewness and kurtosis tests ranged from -1 to +1. Spearman rank test was used for determining correlations.

Results

Patients and controls

Twenty-three healthy subjects were studied to obtain reference values. One subject was excluded from the control group because of thermolabile MTHFR deficiency, with a specific activity of 7.7 nmol CH_2O/mg protein/h and a residual activity after heat inactivation of 15.6%. In the other 22 control subjects a mean (± SD) specific MTHFR activity of 15.6 (± 4.7) nmol CH_2O/mg protein/h (range: 9.1 - 26.6) and a mean (± SD) residual activity after heat inactivation of 55.3 (±

12.0)% (range: 35.9 - 78.3) was observed. In concordance with Kang et al.[9 15 16], thermolabile MTHFR was defined as a specific activity of 50% of the normal mean and a residual activity after heat inactivation of less than 36.0% of the initial activity The hyperhomocysteinemic vascular patients (n = 39) could be divided into two distinct subgroups (Table 5.1). One group (n = 11) with thermolabile MTHFR, showed a mean (± SD) specific activity of approximately 50% of the normal mean: 8 7 (± 2.1) nmol CH_2O/mg protein/h (range: 5.5 - 12.7) and a residual activity after heat inactivation ranging from 0 to 33.0%. The specific MTHFR activity of the thermolabile group showed a small overlap with the control group, so heat inactivation was employed to discriminate between control subjects and patients with thermolabile MTHFR (Figure 5.2). The other group of hyperhomocysteinemic vascular patients (n = 28) had a normal specific activity (± SD) of 21 5 (± 7.2) nmol CH_2O/mg protein/h (range: 10 0 - 39.0) and a residual activity after heat inactivation of 53.8 (± 9.2)% (range: 33.1 - 71.5). The mean (± SD) specific activity of the 29 normohomocysteinemic vascular patients was 20.7 (± 6.5) nmol CH_2O/mg protein/h (range: 9.4 - 33.8) and their mean (± SD) residual activity after heat inactivation was 58.2 (± 10.2)% (range: 43.0 - 82.0) Therefore, among the 29 normohomocysteinemic patients no thermolabile MTHFR activity was observed (Table 5.1), which was significantly different ($p < 0.002$) from the incidence of 11 cases of thermolabile MTHFR among the 39 hyperhomocysteinemic patients.

Cystathionine ß-synthase activity was measured in available fibroblasts of ten patients with mild hyperhomocysteinemia (patients number 3, 10, 13, 16, 18, 20, 23, 25, 27, 33). Their enzyme activities were, respectively, 5.0, 6.5, 9.5, 7.9, 1.6, 5.8, 3.3, 8.0, 6.4 and 6.0 nmol cystathionine/mg protein/h. (normal range 2.3 - 18.2 nmol cystathionine/mg protein/h, N = 12). So, only patient number 18 had activity within the range of obligate heterozygotes for cystathionine ß-synthase deficiency (0.25 - 2.4 nmol cystathionine/mg protein/h, N = 13) Only two studied patients (number 3 and 10) had thermolabile MTHFR and their cystathionine ß-synthase activities were normal.

The 11 patients with thermolabile MTHFR had diverse clinical abnormalities. 4 patients suffered from cerebral, 2 from peripheral and one from coronary artery disease and 4 patients had venous thrombosis. Among the patients with thermolabile MTHFR no correlation was observed between specific MTHFR activity and fasting total homocysteine or total homocysteine after methionine loading. Among all patients with normal MTHFR activity, age did not correlate with specific MTHFR activity or residual MTHFR activity after heat inactivation. The mean (± SD) plasma folic acid level of the patients with thermolabile MTHFR (7.9 ± 2.9 nmol/L) was significantly lower ($p < 0.0002$) than of the reference group (13.6 ± 3 8 nmol/L). However, the plasma folic acid levels of the total group of hyperhomocysteinemic patients (9.2 ± 3.4) was also significantly lower ($p < 0$ 0001) than for the reference group The mean (± SD) vitamin B_{12} concentration of the thermolabile MTHFR deficient patients (222 ± 75 pmol/L) was significantly different ($p = 0$ 052) from that of the reference group (290 ± 115 pmol/L) but the vitamin B_{12} levels of the total group of hyperhomocysteinemic patients (227 ± 84) was also significantly lower ($p < 0.02$) than for the reference group.

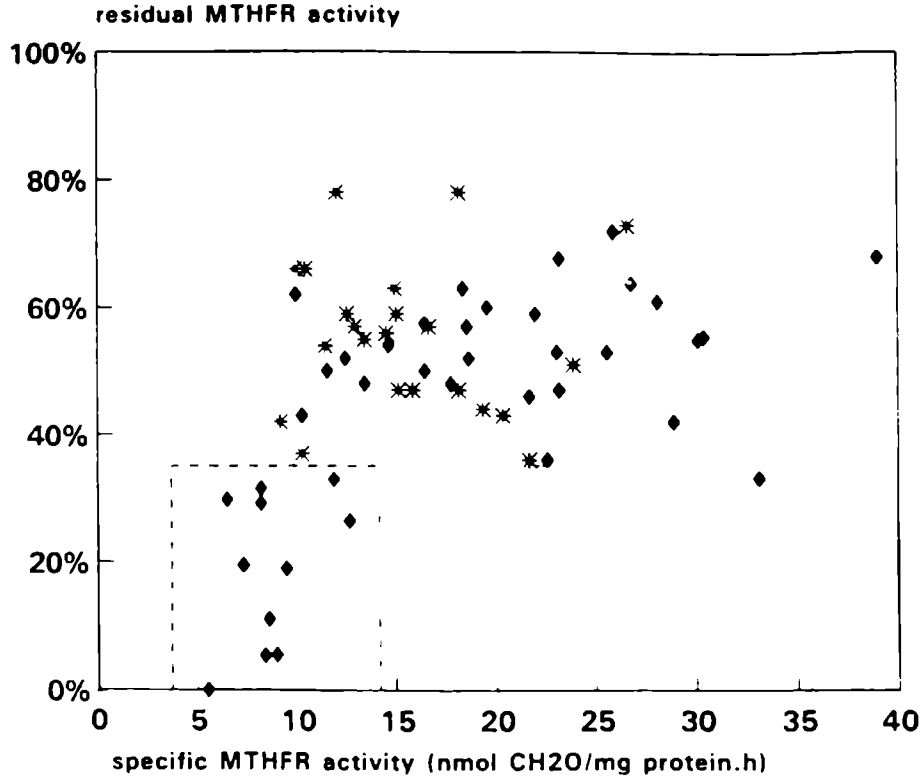

Figure 5.2. Residual MTHFR activity after heat inactivation, versus the specific activity in lymphocytes of hyperhomocysteinemic patients and control subjects. The dashed square contains the patients with thermolabile MTHFR. * = control subjects. ◆ = hyperhomocysteinemic vascular patients.

Assay

After incubation at 37°C, the formaldehyde produced was derivatized by dimedone and the resulting product, formaldemethone, was extracted with toluene. Addition of carrier formaldehyde increased the amount of labeled formaldemethone and therefore the specific MTHFR activity (Figure 5.3). Dimedone and formaldehyde react at a ratio of 2:1[24]. This means that the amount of dimedone must always exceed twice the amount of formaldehyde present. We investigated the derivatization method by optimizing the carrier formaldehyde concentration at a constant dimedone concentration, and optimizing the dimedone concentration at a constant carrier formaldehyde concentration. Optimal derivatization and extraction was achieved by addition of 10 μmoles of carrier formaldehyde and 50 μmoles of dimedone.

Table 5.1. Sex, age, homocysteine concentrations fasting and after loading, specific and residual MTHFR activity after heat inactiviation in patients with premature vascular disease. nd: not determined. [a] Age at the time of this study; [b] Total fasting homocysteine; [c] Total post-load homocysteine.

Patient	Sex	Age[a]	Hcys[b] 0 h	Hcys[c] 6 h	Specific MTHFR activity (nmol CH_2O/mg protein/h)	Residual MTHFR activity(%)
Hyperhomocysteinemic patients with thermolabile MTHFR:						
1	F	28	20	57	9.5	19.4
2	F	52	13	94	5.5	0
3	F	26	19	51	8.4	5.4
4	F	46	13	54	9.0	5.5
5	F	49	17	57	6.5	29.8
6	M	21	31	76	12.7	26.5
7	F	35	18	67	7.3	19.5
8	M	49	32	68	8.2	31.5
9	F	37	27	52	11.9	33.0
10	F	40	17	88	8.6	11.1
11	F	29	38	92	8.2	29.2
	mean	37.5	22.3	68.7	8.7	19.2
	± SD	±10.6	±8.4	±16.4	±2.1	±11.9
Hyperhomocysteinemic patients with normal MTHFR activity:						
12	F	46	10	77	23.1	52.7
13	F	51	11	75	22.6	35.5
14	F	31	27	116	11.6	49.7
15	M	50	20	54	12.5	52.0
16	M	50	21	68	25.9	71.5
17	F	51	14	56	14.7	54.1
18	F	53	nd	75	23.2	67.7
19	M	43	34	71	13.5	47.7
20	F	39	19	50	17.8	48.3
21	F	46	14	63	30.4	55.1
22	F	28	10	54	22.2	59.3
23	F	43	nd	115	18.7	52.2
24	M	29	144	148	10.0	62.0
25	M	40	13	60	26.8	63.4
26	F	33	12	75	30.1	54.6
27	M	42	nd	69	28.1	61.1
28	F	20	30	57	10.3	43.0
29	F	19	16	52	18.6	56.8
30	M	35	26	50	21.7	46.4
31	F	44	15	61	39.0	67.7
32	F	41	13	62	19.6	59.8
33	F	40	nd	84	23.2	47.4
34	M	63	12	61	16.5	49.5
35	M	48	23	52	18.4	63.1
36	F	46	7	59	16.5	57.5
37	F	48	22	74	28.9	42.4
38	F	42	61	145	25.6	52.9
39	F	46	11	67	33.1	33.1
	mean	41.7	24.4	73.2	21.5	53.8
	± SD	± 9.9	± 27.9	± 26.3	± 7.2	± 9.2

Normohomocysteinemic patients:						
40	F	47	13	40	23.7	52.7
41	M	48	15	44	31.3	71.6
42	M	25	14	31	22.1	58.8
43	M	41	11	31	24.6	50.8
44	F	47	13	49	30.5	82.0
45	F	27	11	28	14.8	64.9
46	M	55	12	43	19.6	61.2
47	M	56	14	34	14.0	47.1
48	F	37	10	32	11.4	43.0
49	M	53	8.7	25	33.8	76.2
50	M	41	7.5	22	18.1	49.7
51	M	40	13	37	16.0	61.9
52	F	22	10	27	25.8	60.9
53	M	55	13	38	18.9	64.0
54	F	22	11	28	14.8	65.0
55	F	35	7.8	27	20.5	55.0
56	F	52	17	42	13.0	50.0
57	M	46	15	46	20.0	68.7
58	F	48	13	40	23.7	52.7
59	M	39	13	39	9.4	49.2
60	F	46	8.7	21	29.4	67.7
61	F	39	13	33	17.0	43.2
62	F	31	11	32	21.3	69.2
63	M	42	11	31	24.6	50.8
64	M	47	10	32	28.6	46.3
65	F	51	17	42	13.0	50.0
66	F	34	15	41	26.9	66.2
67	M	56	12	43	19.6	61.2
68	M	57	14	34	14.0	47.1
	mean	43.8	12.2	34.9	20.7	58.2
	± SD	± 12.5	± 2.5	± 7.3	± 6.5	± 10.2

Three different blanks were tested (1) the reaction mixture including the enzyme extract and no incubation at 37°C; (2) the reaction mixture incubated at 37°C without the enzyme extract; (3) the reaction mixture and addition of the enzyme extract after the incubation at 37°C. Blank 1 was inadequate because it resulted in recovery of much less radioactivity than either blank 2 or 3. No difference between blank B and C was observed (data not shown). Blank 2 was used in this study, because it required no enzyme extract.

MTHFR activity depends on the concentration of the cofactor FAD. A maximum activity was observed at 50 μM FAD. In all assays 54 μM FAD was used. Formaldehyde production was linear with incubation time up to 40 min and with amount of enzyme between 10 and 250 μg protein. The incubation time used was 20 min and the amount of enzyme extract added varied from 22 to 150 μg protein. A Km for methyl-THF of 19 μM was observed. Overall, the modifications resulted in higher specific MTHFR activities in lymphocytes than reported elsewhere[9,16-17].

The thermolability of MTHFR in the enzyme extract of pooled lymphocytes of controls was examined by preincubating for 5 min at 37, 40, 46 and 49°C. Incubation at 49°C resulted in a 70% loss, at 46°C in a 53% loss and at 40°C in a 17% loss of activity. Preincubation at 46°C resulted in a 77% loss of enzyme activity at 20 min, 53% loss at 10 min, 43% loss at 5 min and 18% loss of enzyme activity at 2 min (Figure 5.3). Heat inactivation was performed by incubating the enzyme extract for 5 min at 46°C, thus allowing a reliable distinction between thermolabile and thermostable MTHFR in patients and control subjects.

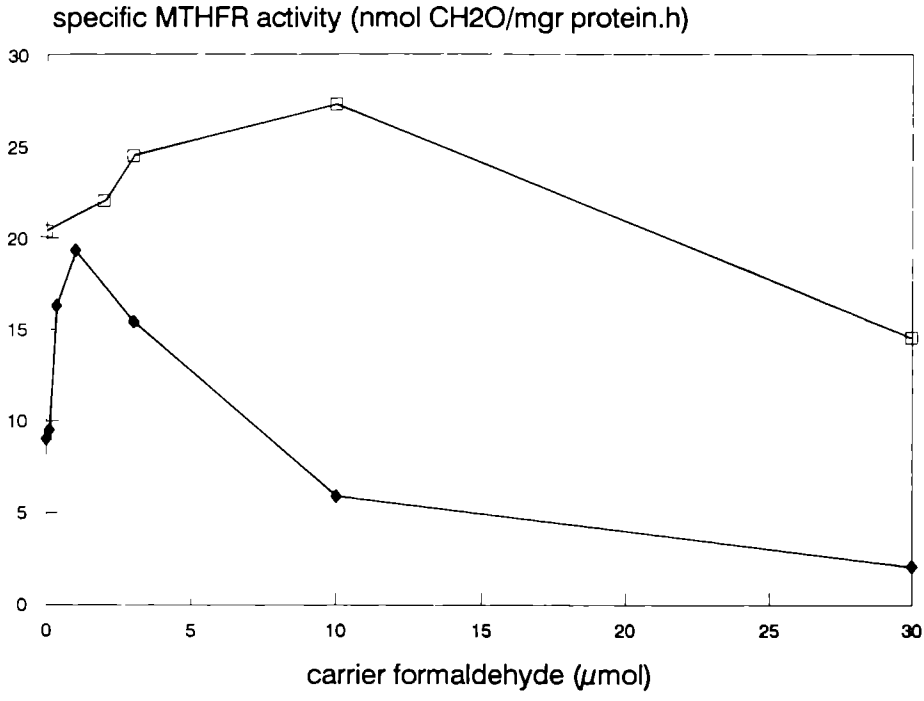

Figure 5.3. Effect of variation of the amount of carrier formaldehyde on the derivatization of labelled formaldehyde with dimedone at two different concentrations. ◆ = 6 μmol dimedone, ■ = 50 μmol dimedone.

Discussion

In this study the thermolabile form of MTHFR was observed in 11 of 39 premature vascular disease patients with mild hyperhomocysteinemia after methionine loading. Nine out of these 11 patients were hyperhomocysteinemic in the fasting state. No thermolabile MTHFR was observed among 29 normohomocysteinemic patients with premature vascular disease and in 23 healthy subjects one case had thermolabile MTHFR. These findings indicate that thermolabile MTHFR is one of the causes leading to mild hyperhomocysteinemia, established by methionine loading in patients with vascular disease.

S-adenosylmethionine is a major regulating compound in homocysteine metabolism because it activates cystathionine ß-synthase and inhibits MTHFR[25]. Plasma homocysteine concentration is supposed to reflect the capacity of homocysteine remethylation in the fasting state because of the low S-adenosylmethionine levels. Whereas, the homocysteine concentration after oral loading with unphysiologic amounts of methionine is believed to evaluate the cystathionine ß-synthase status[2,3]. However, in the present study all vascular patients with the thermolabile form of MTHFR had elevated homocysteine levels after loading and not always in the fasting state. Probably, the folate dependent homocysteine remethylation contributes to homocysteine conversion not only in

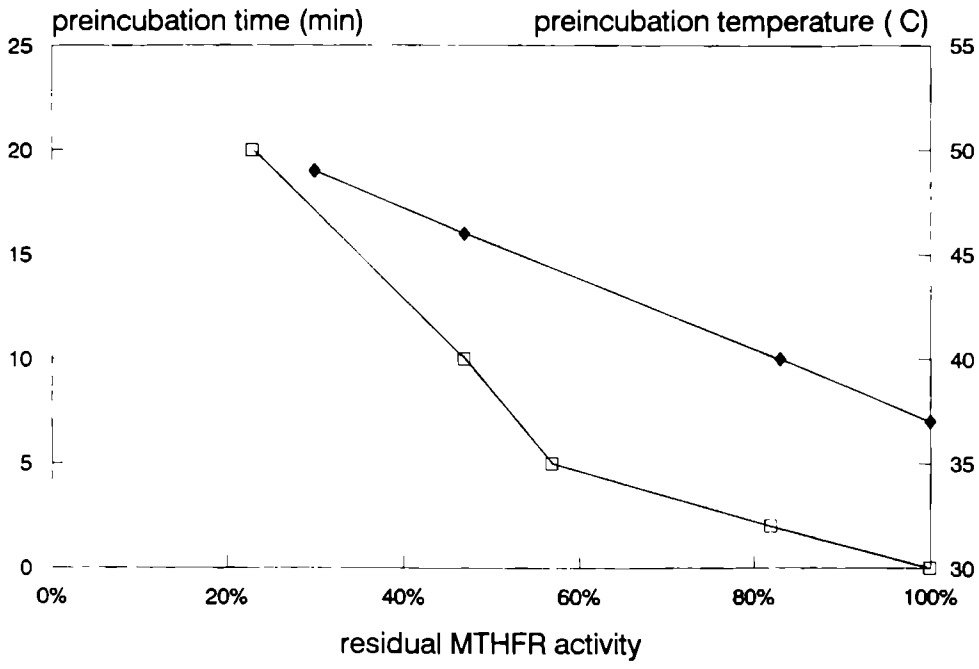

Figure 5.4. Effect of preincubation for 5 min at different temperatures on the residual MTHFR activity (♦). Effect of variation of preincubation time at 46°C on the residual MTHFR activity (■).

the fasting state but also after methionine loading.

Cystathionine ß-synthase activity was assayed in 10 of the 21 vascular patients with mild hyperhomocysteinemia, including two patients (number 3 and 10) with thermolabile MTHFR. All except one (number 18) had normal cystathionine ß-synthase activity. This indicates that decreased cystathionine ß-synthase activity is not the major cause of hyperhomocysteinemia in patients with premature vascular disease.

Kang et al.[9] observed an incidence of 17% of thermolabile MTHFR in a group of 212 patients with coronary arterial disease. Their homocysteine metabolism was not examined by means of a methionine loading test. In a previous study[18] we observed in a large group of patients with diverse forms of vascular disease a prevalence of hyperhomocysteinemia of 24% by using methionine loading tests. The present paper showed that approximately 28% of such hyperhomocysteinemic patients have the thermolabile form of MTHFR. Thus, an incidence of thermolabile MTHFR of 7% among our total group of vascular patients can be calculated which is lower than Kang et al.[9] observed among coronary patients.

The hyperhomocysteinemic patient group consisted of more female than male patients, compared to the normohomocysteinemic patient group. Hyperhomocysteinemic women may be more susceptible to developing vascular disease than hyperhomocysteinemic men[18]. The age of onset of vascular disease

among the thermolabile MTHFR deficient patients varied from 18 to 50 years. The clinical expression of vascular disease among the 11 patients with thermolabile MTHFR was very diverse, including cerebral, peripheral and coronary arterial disease and venous thrombosis. Therefore, thermolabile MTHFR is a risk factor not only for coronary arterial disease, as reported by Kang et al.[9,15] but probably for vascular disease in general.

An incidence of the homozygous form of thermolabile MTHFR of 5% in the normal population is reported[9,15]. This is consistent with our finding of one thermolabile MTHFR deficient subject among 23 healthy subjects. Thus, the heterozygous form must have an incidence of approximately 22% in the normal population. Such a high frequency of this thermolabile MTHFR mutation might be explained by a concomitant beneficial effect of the mutation. Perhaps in times of starvation a reduced MTHFR activity decreases homocysteine remethylation and preserves the available one-carbon moieties of the THF derivatives for the vital synthesis of purines and thymidine.

Severe MTHFR deficiency is very resistant to many homocysteine-lowering and methionine-elevating forms of therapy, including folates, methionine, pyridoxine, vitamin B_{12} and carnitine[5,7]. Betaine appears the best choice of therapy[5,26,28]. It enhances the remethylation of homocysteine to methionine via an alternative pathway (Figure 5.1) and methionine levels increase at the expense of homocysteine. Logical choices of therapy for thermolabile MTHFR deficient patients seem to be folic acid and betaine. Riboflavin could also be an appropriate option since FAD is the cofactor of MTHFR and may stabilize the mutant MTHFR. Two patients with thermolabile MTHFR and mild hyperhomocysteinemia showed a dramatic decrease of total homocysteine concentration after folic acid therapy[16]. Folic acid administration may increase the concentrations of MTHF which is the substrate of MTHFR.

On the relationship between vascular disease and mild hyperhomocysteinemia due to a 50% reduction in MTHFR activity the following considerations can be made: (1) in the vascular wall THF dependent remethylation constitutes the primary mechanism for homocysteine conversion. The betaine-homocysteine methyltransferase is present only in the liver and maybe in the kidney and the Km of cystathionine ß-synthase is at least ten times higher than that of the enzymes involved in THF dependent homocysteine remethylation[25]; (2) mild hyperhomocysteinemia is likely caused by a combination of genetic and environmental factors, including methionine, betaine, choline, folate, vitamin B_6 and B_{12}; (3) in addition, although thermolabile MTHFR and hyperhomocysteinemia are correlated, and hyperhomocysteinemia is a risk factor for vascular disease, some other effect of thermolabile MTHFR besides its resultant hyperhomocysteinemia may put patients at risk for vascular disease.

In conclusion, thermolabile MTHFR is the most likely cause of abnormal homocysteine metabolism in approximately 28% of the vascular patients with mild hyperhomocysteinemia.

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References

1. Brattström L, Lindgren A. Hyperhomocysteinemia as a risk factor for stroke. Neur Res 1992;14:81-84.
2. Kang SS, Wong PWK, Malinow MR. Hyperhomocyst(e)inemia as a risk factor for occlusive vascular disease. Annu Rev Nutr 1992;12:279-298.
3. Ueland PM, Refsum H, Brattström L. Plasma homocysteine and cardiovascular disease. In: Francis RB (ed). Artherosclerotic cardiovascular disease, hemostasis, and endothelial function. Marcel Dekker Inc., New York, 1992;183-236.
4. Blom HJ, van der Molen E. Pathobiochemical implications of hyperhomocysteinemia. Fibrinolysis 1994;8:86-87.
5. Rosenblatt DS. Inherited disorders of folate transport and metabolism. In: Scriver CR, Beaudet AL, Sly WS, Valle D (eds). The metabolic basis of inherited disease. McGraw-Hill Inc., New York, 1989;2049-2064.
6. Wada Y, Narisawa K, Arakawa T. Infantile type of homocystinuria with 5,10-methylenetetrahydrofolate reductase deficiency. Monogr Hum Genet 1978;9:140-146.
7. Harpey JP, Rosenblatt DS, Cooper BA, Le Moël G, Roy C, Lafourcade J. Homocystinuria caused by 5,10-methylenetetrahydrofolate reductase deficiency: A case in an infant responding to methionine, folinic acid, pyridoxine and vitamin B_{12} therapy. J Pediatr 1981;98:275-278.
8. Wendel U, Claussen U, Diekmann E. Prenatal diagnosis for methylenetetrahydrofolate reductase deficiency. J Pediatr 1983;102:938-940.
9. Kang SS, Wong PWK, Susmano A, Sora J, Norusis M, Ruggie N. Thermolabile methylenetetrahydrofolate reductase: An inherited risk factor for coronary artery disease. Am J Hum Genet 1991;48:536-545.
10. Boers GHJ, Fowler B, Smals AGH, Trijbels JMF, Leermakers AI, Kleijer WJ, Kloppenborg PWC. Improved identification of heterozygotes for homocystinuria due to cystathionine synthase deficiency by the combination of methionine loading and enzyme determination in cultured fibroblasts. Hum Genet 1985;69:164-169.
11. Boers GHJ, Smals AGH, Trijbels JMF, Fowler B, Bakkeren JAJM, Schoonderwaldt HC, Kleijer WJ, Kloppenborg PWC. Heterozygosity for homocystinuria in premature peripheral and cerebral occlusive arterial disease. N Engl J Med 1985;313:709-715.
12. Clarke R, Daly L, Robinson K, Naughten E, Cahalane S, Fowler B, Graham I. Hyperhomocysteinemia: an independent risk factor for vascular disease. N Eng J Med 1991;324:1149-1155.
13. Mudd SH, Havlik R, Levy HL, McKusick VA, Feinleib M. A study of cardiovascular risk in heterozygotes for homocystinuria. Am J Hum Genet 1981;33:883-893.
14. Daly L, Robinson K, Tan KS, Graham I. Hyperhomocysteinemia: a metabolic risk factor for coronary heart disease determined by both genetic and environmental influences? Quart J Med 1993;86:685-689.
15. Kang SS, Wong PWK, Zhou J, Sora J, Lessick M, Ruggie N, Grcevich G. Thermolabile methylenetetrahydrofolate reductase in patients with coronary artery disease. Metabolism 1988;37:611-613.
16. Kang SS, Zhou J, Wong PWK, Kowalisyn J, Strokosch G. Intermediate homocysteinemia: A thermolabile variant of methylenetetrahydrofolate reductase. Am J Hum Genet 1988;43:414-421.
17. Kang SS, Wong PWK, Bock HGO, Horwitz A, Grix A. Intermediate hyperhomocysteinemia resulting from compound heterozygosity of methylenetetrahydrofolate reductase mutations. Am J Hum Genet 1991;48:546-551.
18. Franken DG, Boers GHJ, Blom HJ, Trijbels JMF, Kloppenborg PWC. Treatment of mild hyperhomocysteinemia in vascular disease patients.

Arterioscl Thromb 1994;14:465-470.
19. Jondal M, Holm G, Wigzell H. Surface markers on human T and B lymphocytes. I. A large population of lymphocytes forming nonimmune rosettes with sheep red blood cells. J Exp Med 1972;136:207-215.
20. Rosenblatt DS, Erbe RW. Methylenetetrahydrofolate reductase in cultured human cells. II. Genetic and biochemical studies of methylenetetrahydrofolate reductase deficiency. Pediatr Res 1979;11:1141-1143.
21. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. J Biol Chem 1951;193:265-275.
22. Fowler B, Kraus J, Packman S, Rosenberg LE. Evidence for three distinct classes of cystathionine ß-synthase mutants in cultured fibroblasts. J Clin Invest 1978;61:645-653.
23. te Poele-Pothoff MTWB, van den Berg M, Franken DG, Boers GHJ, Jakobs C, de Kroon IFI, Eskes TKAB, Trijbels JMF, Blom HJ. Three different methods for the determination of total homocysteine in plasma. Ann Clin Biochem 1995;32:218-220.
24. Ruyter IE. Release of formaldehyde form denture base polymers. Acta Odontol Scand 1980;38:17-27.
25. Finkelstein JD. Methionine metabolism in mammals. J Nutr 1990;1:228-237.
26. Benevenga NJ. Betaine in treatment of homocystinuria. N Eng J Med 1984;310:265-266.
27. Wendel U, Bremer HJ. Betaine in treatment of homocystinuria due to 5,10-methylenetetrahydrofolate reductase deficiency. Eur J Pediatr 1984;142:147-150.
28. Hyland K, Smith I, Bottiglieri T, Perry J, Wendel U, Clayton PT, Leonard JV. Demyelination and decreased S-adenosyl-methionine in 5,10-methylenetetrahydrofolate reductase deficiency. Neurology 1988;38:459-462.

FAMILIAL CEREBROVASCULAR ACCIDENTS DUE TO CONCOMITANT HYPERHOMOCYSTEINEMIA AND PROTEIN C DEFICIENCY TYPE 1.

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Abstract

Background and purpose: Hyperhomocysteinemia as well as protein C deficiency are risk factors for thromboembolism. Hyperhomocysteinemia have been reported to inhibit the expression of thrombomodulin and to inactivate both thrombomodulin and protein C irreversibly, leading to a decreased protein C activity.

Case: In a 16-year-old girl, who developed a sinus sagittalis thrombosis, and in her father, who experienced a transient ischemic attack, both hyperhomocysteinemia and protein C deficiency type 1 were present. Protein C deficiency alone was found in one of the two sisters who was without any clinical vascular history.

Conclusions: In this family with independently inherited hyperhomocysteinemia and protein C deficiency, clinical cerebrovascular disease occurred only in those members with a combination of both risk factors, suggesting a synergistic interaction between these thrombogenic risk factors.

Introduction

Mild hyperhomocysteinemia has come to be recognized as an independent risk factor for thromboembolic and premature arteriosclerotic complications[1-5]. Although the mode of inheritance is not established, there is evidence that mild hyperhomocysteinemia is influenced by genetic factors[6].

Protein C deficiency type 1 is a disorder with autosomal dominant inheritance with variable penetrance, in which protein C activity as well as protein C antigen is decreased[7,8]. The conversion of protein C into activated protein C is enhanced by the cofactor thrombomodulin. A deficiency of the anticoagulant protein C, both in its hereditary and acquired forms, is also recognized as an independent risk factor for venous and arterial thrombosis[9,10].

Homocysteine has been reported in in vitro studies to inhibit thrombomodulin expression on endothelial cell surface and to inactivate both thrombomodulin and protein C irreversibly[11,12]. Thus, hyperhomocysteinemia as well as protein C deficiency are not only both independent risk factors for thrombotic and thromboembolic complications, but furthermore, hyperhomocysteinemia may exaggerate the decrease of the protein C activity in case of protein C deficiency.

In this report, we present as the history of a teenage girl from a family with inherited hyperhomocysteinemia as well as protein C deficiency type 1.

Case report

A 16-year-old girl born to non-consanguineous parents was admitted to our hospital with vomiting, fever, and nuchal rigidity. Penicillin was administrated because bronchitis was suspected. After 3 days she developed headache, dysphasia, dysarthria, and a right-sided hemiparesis. Venous and arterial digital subtraction angiography revealed thrombosis of the superior and inferior sagittal sinus and straight sinus. Additionally, a moderate hypercholesterolemia (7,7 mmol/L; reference value $\leq$ 6,0 mmol/L) was detected. She had been smoking about 2 to 5 cigarettes daily for 3 years, and used sub-50 oral contraceptives, containing 35 micrograms ethinylestradiol and 2 milligrams cyproteronacetate (Diane-35^{R}) for 5 months.

The diagnosis hyperhomocysteinemia in the index case and family members was made by means of a standardized methionine loading test as reported previously[13]. The plasma total homocysteine concentration immediately before and 6 hours after the methionine loading was analyzed by high performance liquid chromatography[14]. Protein C activity and antigen screening in these persons was performed and analyzed by a previously described method[15].

Both hyperhomocysteinemia (19 before and 60 μmol/L after load; reference mean $\pm$ 2 SD for premenopausal women is 5 to 15 μmol/L and 15 to 49 μmol/L, respectively) and protein C deficiency type 1 (protein C activity of 33%, protein C antigen of 45%; reference values are >60% and >70%, respectively) were present in the index patient.

The patient's 43-years-old father had smoked 20 cigarettes daily since the age of 14 years, and had a history of a transient ischemic attack at the age of 40. In addition, both hyperhomocysteinemia (34 μmol/L before and 71 μmol/L after load; reference mean $\pm$ 2 SD for men 7 to 17 μmol/L and 20 to 51 μmol/L, respectively) and protein C deficiency type 1 (protein C activity of 60%, protein C antigen of 58%) were established. The patient's 39-years-old mother, who had

smoked from age 14 to 33, and two younger sisters (15 and 13 years of age, respectively), both without smoking habits, were without history of vascular complications, and did not show hyperhomocysteinemia after methionine loading (13, 13 and 12 μmol/L before load, respectively, and 35, 23 and 24 μmol/L after load, respectively). The protein C activity and protein C antigen in her mother (95% and not measured, respectively) and in one of the two sisters (68% and 74%, respectively) appeared to be normal. However, protein C deficiency type 1 was present in the other sister (protein C activity of 41%, protein C antigen of 47%).

With the exception of the use of tobacco in both parents, there were no further predisposing factors for vascular disease present in the parents and sisters. In particular, no hyperlipoproteinemia, diabetes or high blood pressure was noted.

Discussion

Cerebral venous thrombosis is often associated with sepsis, dehydration, polycythemia, malignancies, post partum state, use of oral contraceptives, antithrombin III deficiency, systemic lupus erythematodes, head injury and Behçet's syndrome[16]. Both hyperhomocysteinemia and protein C deficiency type 1 also are risk factors for thrombosis[4 5 8]. In the studied family both states were independently inherited, and clinical thrombosis occurred only in those family members with hyperhomocysteinemia as well as protein C deficiency type 1. In these cases the elevated homocysteine concentration may have decreased the deficient protein C activity even more, resulting in a very high risk for thrombosis.

Hyperhomocysteinemia was treated with vitamin B_6, 250 mg daily, and the homocysteine concentration decreased to 15 and 37 μmol/L in the index patient and to 24 and 57 μmol/L in her father before and after load, respectively. The thrombotic tendency caused by the protein C deficiency was attenuated by anticoagulant therapy in the 16-year-old girl and by salicylate administration in her father While on this treatment, no further complications as a result of their proneness to thrombotic events have occurred during the last 2 years.

The basis of the hyperhomocysteinemia in this family is unclear. Before the performance of the methionine loading test, secondary causes of mild hyperhomocysteinemia had been excluded such as vitamin B_6, B_{12} and folic acid deficiencies, and failure of liver and renal functions. Possible genetic defects in methionine metabolism leading to mild hyperhomocysteinemia are heterozygosity for cystathionine synthase deficiency or homozygosity for thermolabile 5,10-methylenetetrahydrofolate reductase[1 2 17]. The determination of these enzymes activities was not performed on a routine base and are not available in these hyperhomocysteinemic family members The beneficial effect of homocysteine-lowering treatment with vitamin B_6 might suggest the presence of the heterozygosity for cystathionine synthase deficiency in this particular family.

We conclude that screening for hyperhomocysteinemia as well as for protein C deficiency in patients presenting with thromboembolic events of unknown origin is recommendable because both factors may lead independently to thrombosis. Moreover, both factors may have specifically a possible synergistic interaction as is indicated in previous in vitro studies[11 12]

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References

1. Boers GHJ, Smals AGH, Trijbels JMF, Fowler B, Bakkeren JAJM, Schoonderwaldt HC, Kleyer WJ, Kloppenborg PWC. Heterozygosity for homocystinuria in premature peripheral and cerebral occlusive arterial disease. N Engl J Med 1985;313:709-715.
2. Clarke R, Daly L, Robinson K, Naughten E, Cahalane S, Fowler B, Graham I. Hyperhomocysteinemia: an independent risk factor for vascular disease. N Engl J Med 1991;324:1149-1155.
3. Coull BM, Malinow MR, Beamer N, Sexton G, Nordt F, deGarmo P. Elevated plasma homocyst(e)ine concentration as a possible independent risk factor for stroke. Stroke 1990;21:572-576.
4. Brattström L, Tengborn L, Lagerstedt C, Israelsson B, Hultberg B. Plasma homocysteine in venous thromboembolism. Haemostasis 1991;21:51-57.
5. Bienvenu T, Ankri A, Chadefaux B, Kamoun P. Dosage de L'homocystéine plasmatique dans l'exploration des thromboses du sujet jeune. Presse Méd 1991;21:985-988.
6. Genest JJ, McNamara JR, Upson B, Salem DN, Ordovas JM, Schaefer EJ, Malinow MR. Prevalence of familial hyperhomocyst(e)inemia in men with premature coronary artery disease. Arteriosclerosis and Thrombosis 1991;11:1129-1136.
7. Broekmans AW, Veltkamp JJ, Bertina RM. Congenital protein C deficiency and venous thromboembolism: A study of three Dutch families. N Engl J Med 1983;309:340-344.
8. Clouse LH, Comp PC. The regulation of hemostasis: The protein C system. N Engl J Med 1986;314:1298-1304.
9. Hedner U, Davie EW. Blood and blood-forming tissue. In: Scriver CR, Beaudet AL, Sly WS, Vall D (eds). The metabolic basis of inherited disease. McGraw-Hill Inc., New York, 1989;2107-2134.
10. Coller BS, Owen J, Jesty J, Horowitz D, Reitmann MJ, Spear J, Yeh T, Comp PC. Deficiency of plasma protein S, protein C or antithrombin III and arterial thrombosis. Arteriosclerosis 1987;7:456-461.
11. Lentz SR, Sadler JE. Inhibition of thrombomodulin surface expression and protein C activation by the thrombogenic agent homocysteine. J Clin Invest 1991;88:1906-1914.
12. Hayashi T, Honda G, Suzuki K. An atherogenic stimulus homocysteine inhibits cofactor activity of thrombomodulin and enhances thromomodulin expression in human umbilical vein endothelial cells. Blood 1992;11:2930-2936.
13. Boers GHJ, Fowler B, Smals AGH, Trijbels JMF, Leermakers AI, Kleyer WJ, Kloppenborg PWC. Improved identification of heterozygotes for homocystinuria due to cystathionine synthase deficiency by the combination of methionine loading and enzyme determination in cultured fibroblasts. Hum Gent 1985;69:164-169.
14. Fiskerstrand T, Refsum H, Kvalheim G, Ueland PM. Homocysteine and other thiols determination in plasma and urine: automation and sample stability. Clin Chem 1993;39:263-271.
15. Martinoli JL, Stocker K. Fast functional protein C assay using protac, a novel protein C activator. Thromb Research 1986;43:253-264.
16. Bousser M-G, Chiras J, Bories J, Castaigne P. Cerebral venous thrombosis - A review of 38 cases. Stroke 1985;16:199-213.
17. Kang S-S, Wong PWK, Susmano A, Sora J, Norusis M, Ruggie N. Thermolabile methylenetetrahydrofolate reductase: an inherited risk factor for coronary artery disease. Am J Hum Genet 1991;48:536-545.

PART 2

BIOCHEMICAL EFFECT OF TREATMENT OF HYPERHOMOCYSTEINEMIA

TREATMENT OF MILD HYPERHOMOCYSTEINEMIA

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Abstract

A review of studies is presented on the effect of homocysteine-lowering therapy in mildly hyperhomocysteinemic vascular patients. Pooled data showed that it is possible to decrease elevated fasting plasma homocysteine levels as much as a mean of 40% by the use of a low dose of 0.65 mg folic acid daily. A higher dose, i.e. 5 mg daily, resulted in a slightly larger reduction of about 50%; 10 mg folic acid daily had no extra effect. Vitamin B_{12} in a low oral dose of 0.400 mg daily is advisable to add because of its modest homocysteine-lowering effect but more to avoid folic acid-refractoriness in cases of vitamin B_{12} deficiency and to prohibit the development of neuropathy due to unrecognized perniciosa. Vitamin B_6 does not affect fasting hyperhomocysteinemia.

Elevated post-load homocysteine levels as established by means of methionine loading, can be normalized in more than 90% of the patients by treatment with 100 mg vitamin B_6 plus 5 mg folic acid daily. This combination showed a mean reduction of post-load concentrations of about 50%, somewhat higher than the 40% obtained by vitamin B_6 as single agent. The efficacy of lower doses of vitamins has not been studied.

Introduction

In line with the first report by Sardharwalla et al.[1] of mildly elevated homocysteine levels after an oral methionine load in obligate heterozygotes for cystathionine synthase deficiency, it has been proposed that the comparably mild hyperhomocysteinemia detected in about 25% of vascular patients also originates from heterozygosity for this specific enzyme defect[2]. In the 2 reports presenting determinations of cystathionine synthase activity in cultured fibroblasts from hyperhomocysteinemic vascular patients, an intermediate enzyme deficiency, indeed, has been confirmed in the majority of these patients[3,4]. In recent years, however, it has become increasingly clear that identification of hyperhomocysteinemic patients with vascular disease as heterozygotes for cystathionine synthase deficiency has not been convincing so far[6]. More recent determinations of enzyme assays in such patients show that cystathionine synthase activity is defective only in sporadic cases[5,7] and that thermolability of 5,10-methylenetetrahydrolate reductase is a far more common genetic defect among these patients[5,8]. Notwithstanding its occurrence up to 25% in some groups of hyperhomocysteinemic vascular patients[5], this prevalence still does not seem high enough to predict a predominant role of this defect among possible causes of mild hyperhomocysteinemia in vascular patients. The total range of these possible causes either genetical or environmental is not at all completely explored and, as a consequence, the design of homocysteine-lowering treatment with maximal efficacy may be at the moment illusory for the whole group of hyperhomocysteinemic patients.

The earlier assumption - albeit incorrect in retrospective - of heterozygous cystathionine synthase deficiency as the major cause of mild hyperhomocysteinemia in vascular patients, has had created the comfortable situation in which it was justified to deduce the guidelines for homocysteine-lowering intervention in these 'heterozygotes' from the extended experience in the homozygous condition. In homozygotes for cystathionine synthase deficiency the severely elevated homocysteine levels in the blood can be lowered or even normalized by treatment with high doses of vitamin B_6 which as pyridoxine phosphate is cofactor in the specific enzymatic reaction. The majority of the adult patients responds beneficially to such treatment and in case of absent or insufficient effect additional supplementation of cofactors or cosubstrates for the remethylation of homocysteine into methionine such as folic acid, vitamin B_{12}, and betaine (= trimethylglycine) will be favourable[9]. The ultimate therapeutical option of dietary methionine restriction is therefore seldom required. Analogously to this established therapeutical regimen in severely hyperhomocysteinemic patients, homocysteine-lowering intervention has also been carried out in mildly hyperhomocysteinemic vascular patients.

Review of previous reports

The effect of vitamin B_6 and folic acid

The first data on biochemical responsiveness of vascular patients with mild hyperhomocysteinemia to potentially homocysteine-lowering treatment were presented by **Boers** in 1988[10]. Out of 32 of suchlike patients 26 (81%) normalized their pathological hyperhomocysteinemia after methionine loading on treatment with vitamin B_6, 250 mg daily for 6 weeks, supplemented with folic acid, 5 mg

daily, only in cases in which folate deficiency was present. The remaining 6 patients, unresponsive to vitamin B_6, showed a normalization of the post-load hyperhomocysteinemia after the start with betaine, 6 gram daily. In these patients fasting homocysteine levels and their response upon treatment were not presented because the concentrations measured as free homocysteine and not as total homocysteine what became possible in later years, were indiscriminately low[11]. The dosages of the prescribed substances had been deduced arbitrarily from the established regimen in homozygotes for cystathionine synthase deficiency. It was considered that in these 'heterozygotes' with less severe hyperhomocysteinemia, a lower dose of vitamin B_6 should be sufficiently potent. Furthermore, the risk of sensory peripheral neuropathy which had been reported as a sporadical adverse effect in non-hyperhomocysteinemic users of high doses up to 6 gram daily, had to be minimalized[11 12]. The dosages of folic acid and betaine had not been adjusted because no side effects of these substances are known[11].

Brattström et al.[13] in 1990 reported in 20 hyperhomocysteinemic vascular patients the homocysteine-lowering effect of treatment with 240 mg vitamin B_6 daily followed by a period of 2 weeks with 10 mg folic acid daily in addition. After vitamin B_6 alone the mean basal concentration remained unchanged but the mean increase of the levels after methionine loading decreased by 26%. The combined treatment, however, resulted in a mean reduction of the basal levels by 53% and of the post-load increase by 39%. In how many patients the hyperhomocysteinemia had been normalized while on treatment is not indicated.

Dudman and coworkers in 1993 showed about similar results with a lower supplement of 100 mg vitamin B_6 daily which induced a decrease of the post-load homocysteine levels of 35% in hyperhomocysteinemic patients with vascular disease[14]. Folic acid as the sole therapy, 5 mg daily, resulted in a reduction of 45% and the combination of vitamin B_6 and folic acid, 100 mg respectively 5 mg daily, in a decrease of 51% of the post-load levels. The numbers of treated patients in each group were small, however, i.e. 5, 6, and 12 respectively, but it could be calculated that these effects did not differ statistically significantly from each other. In this study free homocysteine has been determined in the blood. The effects of the various treatments on the fasting levels are not available.

Franken et al.[11] presented in 1994 retrospective data on homocysteine-lowering treatment in a large group of hyperhomocysteinemic vascular patients. During the period 1980 to 1990 a total of 421 young arteriosclerotic patients had been screened for mild hyperhomocysteinemia by means of a methionine loading test Fasting homocysteine levels were not considered because the determination of the concentration of free instead of total homocysteine left the basal levels unmanageably low. Out of the 421 patients 100 proved to be hyperhomocysteinemic and 82 of these had been treated with 250 mg vitamin B_6 daily. After 6 weeks of treatment post-load homocysteine levels again were assessed and 56% of the treated patients showed a normalization of their hyperhomocysteinemia, 20% had an intermediate response at least 60% reduction of the pathological elevation of the post-load levels and 24% a poor response of less than 60% reduction or no response at all. The mean reduction of the post-load levels was 39%, higher than the 26% reduction observed by Brattstrom et al.[13] after not more than 2 weeks of treatment with nearly the same dose of vitamin B_6 but about equal as obtained by Dudman et al [14] in long-term treatment with a lower

dose of 100 mg vitamin B_6 daily. The non-normalized patients after 6 weeks of therapy with vitamin B_6 were further treated with folic acid, 5 mg daily, and/or betaine which resulted in 95% in a normalization as yet, whereas vitamin B_6 was continued only if it had had at least some intermediate response.

Also in 1994 **van den Berg** et al.[15] published the results of screening of mild hyperhomocysteinemia in 309 young vascular patients of whom 23% showed abnormal homocysteine levels after methionine loading while only half of these detected patients presented concomitantly fasting hyperhomocysteinemia. In all hyperhomocysteinemic patients the prescribed treatment was identical, i.e. 250 mg vitamin B_6 plus 5 mg folic acid daily for 6 weeks. Post-load hyperhomocysteinemia normalized in 92% of the treated patients and fasting hyperhomocysteinemia, if concomitantly present, in 91%. The mean reduction of the post-load homocysteine concentrations was 48%, remarkably similar as the decrease which Dudman[14] observed in his 12 treated patients after the use of only 100 mg of vitamin B_6 in combination with the same dose of 5 mg folic acid daily. However, the decrease of the post-load levels was higher than the 39% as obtained by Franken et al.[11] after an equal period of therapy with the same dose of vitamin B_6 alone, and also higher than the 39% reduction as achieved by Brattstrom et al.[13] with the same combination but only for 2 weeks. The mean decrease of the fasting levels, i.e. 51%, equalled that from Brattstrom's study.

Ubbink and coworkers presented in 1994 the results of a placebo-controlled intervention to lower fasting homocysteine levels[16]. From a group of 2788 ambulatory men, aged between 20 and 73 years, who were referred for routine medical investigations for life insurance purposes, they recruited 100 participants with a fasting homocysteine level exceeding the normal reference range, i.e. above 16.3 μmol/L. These persons were randomly assigned to one of 5 groups, 20 for each. Group A received placebo tablets, group B folic acid tablets (0.65 mg), group C vitamin B_6 tablets (10 mg), group D cyanocobalamin tablets (0.4 mg) and group E a tablet containing all these vitamins in the above-indicated doses as a combination. After the use of these tablets in a dose of 1 daily for 6 weeks, the fasting blood levels of homocysteine showed no change in group A, a reduction of 42% ($p < 0.001$) in group B, 5% (not significant) in group C, 15% ($p < 0.01$) in group D, and 50% ($p < 0.001$) in group E. The largest reduction caused by the supplement containing all 3 vitamins (group E) differed not statistically significantly from that obtained by folate supplementation alone (group B). Therefore, the conclusion of the authors is that the homocysteine-lowering effect of the multivitamin combination is mainly originating from its folic acid component. Notwithstanding that, it was remarkable that only in the group with combined vitamin supplementation 100% of the participants responded with a reduction of their circulating homocysteine concentration whereas in the group with folic acid as a single agent therapy 10% of the persons failed to respond.

Only very recently in 1995, **Landgren** et al.[17] studied the effect of the use of various doses of folic acid on the fasting homocysteine level in 53 patients after a myocardial infarction. Sixteen, i.e 30%, showed moderate fasting hyperhomocysteinemia with levels above 17.3 μmol/L. The patients were randomly allocated in an open study either to no treatment ($n = 20$), to treatment with 2.5 mg ($n = 17$), or 10 mg ($n = 16$) of folic acid daily. After 6 weeks the no-treatment group even showed a subtile still significant increase (+4%) of the plasma

homocysteine levels, whereas the groups which took 2.5 mg as well as the group which used 10 mg folic acid daily presented a mean reduction in their fasting homocysteine level of 27%. In the 12 included patients with hyperhomocysteinemia the mean reduction was more marked than in the normohomocysteinemic ones, i.e. about 37%, but this effect was not superior to that obtained by Ubbink[16] with a dose of folic acid as low as 0.65 mg daily.

The effect of vitamin B_{12}

Both in healthy subjects[18,19] and in hyperhomocysteinemic vascular patients[13,17] there are indications that vitamin B_{12} supplementation is only effective as a homocysteine-lowering treatment in cases with low or low-normal vitamin B_{12} blood levels, which condition leads to elevated fasting homocysteine concentration but not to exaggerated rises after methionine loading. Nevertheless, Ubbink et al.[16] obtained by oral supplementation of 0.4 mg cyanocobalamin daily a modest, still significant decline of the mean fasting homocysteine level of 15% in 20 hyperhomocysteinemic subjects. Some of their participants, however, could have had low vitamin B_{12} blood levels because the pretreatment vitamin nutritional status was unknown.

Apart from this - albeit moderate - ability to lower fasting homocysteine levels, additional arguments to include vitamin B_{12} in a homocysteine-lowering regimen are the fact that folate supplementation alone will not correct hyperhomocysteinemia if it is the result of low vitamin B_{12} status[20], and that the use of folic acid as a sole treatment will deteriorate a silent neuropathy due to unrecognized pernicious anaemia. A daily oral dose of at least 0.4 mg cyanocobalamin should be sufficient to guarantee adequate resorption of vitamin B_{12} from the gastrointestinal tract even in perniciosa patients[16].

The effect of betaine

So far, the effect of the use of betaine on the homocysteine levels in hyperhomocysteinemic vascular patients, has been reported only in sporadic cases. When it has been prescribed because of an insufficient response of the post-load hyperhomocysteinemia to first choice treatments like vitamin B_6 and/or folic acid, it could always normalize the excess rise of homocysteine in a dose of 6 g daily[10,11,15]. Dudman et al.[11] showed that out of 9 hyperhomocysteinemic vascular patients 7 yielded a mean decrease of their post-load homocysteine levels of 38% after the use of 1.68 g betaine daily for one week, which response was about similar to that obtained by vitamin B_6 or folic acid in these patients. The 2 non-responders to betaine did not react to any homocysteine-lowering treatment. Effects of betaine treatment upon fasting hyperhomocysteinemia have not been mentioned in the various reports.

Despite these promising, although very scattered reports of betaine therapy as homocysteine-lowering agent, the prescription of this substance on a large scale in hyperhomocysteinemic vascular patients will meet difficulties in many countries because it is a non-registered drug. Long-term treatment in many homozygotes for cystathionine synthase deficiency did not reveal any adverse effect of the substance, however[9].

What to treat: fasting or post-load hyperhomocysteinemia?

It is beyond the scope of this paper to decide at this stage if detection of mild hyperhomocysteinemia in an individual vascular patient requires the performance of a methionine loading test or that the determination of a fasting plasma level should be sufficiently sensitive. In our experience, in as much as 30 to 50% of post-load hyperhomocysteinemic vascular patients there is no concomitant fasting hyperhomocysteinemia[15,21]. From pooled data on about 500 patients with arteriosclerotic vascular disease under the age of 60 years and in about 300 control subjects, an odds ratio of 13.0 (95% confidence interval 5.9 - 28.1) can be calculated as the estimate of the relative cardiovascular risk in subjects with an abnormal response to methionine loading compared to normal responders[22]. On the other hand, two recent studies assessed prospectively the risk of coronary disease in 21,000 persons from the general population[23] and in about 15,000 physicians who participated in the US-Physicians' Health Study[24]. In the former study, a relative risk was shown of 1.32 (95% confidence interval 1.05-1.65) and in the latter one of 1.18 (95% confidence interval 1.03-1.36) for each one-standard deviation increase of homocysteine above the mean. Therefore, the relative risk of cardiovascular disease constituted by abnormal post-load homocysteine seems to be higher than that associated with moderately elevated fasting levels. However, more insights are expected in the next future from the publication of the results of a large case-control study performed in more than 800 prematurely arteriosclerotic patients and as much matched control subjects recruited from several European countries[25].

It could be argued that loading with an excess of methionine to screen for hyperhomocysteinemia in vascular patients is reflecting an unphysiological state because the thereby induced increase of S-adenosylmethionine concentration is directing the homocysteine metabolism disproportionally towards the transsulphuration pathway due to stimulation of activity of cystathionine synthase and inhibition of methylenetetrahydrofolate reductase activity by this intermediate[29]. Such mechanism has also been held responsible for the finding that vitamin B_6, cofactor in the cystathionine synthase reaction, is effective in lowering a post-load hyperhomocysteinemia but does not affect a fasting elevated homocysteine level.

On the other hand, the intraindividual day-to-day variation in fasting homocysteine levels is disturbingly wide, up to 25%, which should represent also a day-to-day variation in risk of developing myocardial infarction of about 25%[27]! One of the reasons for this variation could be dietary changes during the day preceding the fasting venous samplings because it has been proven that a protein-rich meal may affect plasma homocysteine for at least 8 hours[27]. A post-load homocysteine determination, 4 or 6 hours after the methionine intake in the fasting state in the morning, will have a distance in time from the last protein-containing meal of at least 16 hours and during the test itself the protocol prohibits protein intake. Therefore, less intraindividual variation in post-load homocysteine levels is probable although not proven so far.

A very serious argument against the use of the post-load homocysteine level as criterion of hyperhomocysteinemia will be the fact that the methionine loading test is too laborious and expensive to include in the design of epidemiological studies with large numbers of participants. But already in diagnostic procedures in

individual vascular patients, the practical concerns and high costs of establishing post-load homocysteine levels will make most probably the determination of fasting levels the only accessible when in the future screening for this risk factor will become a routine procedure on a large scale. If so, more studies have to be done to standardize the sampling of fasting levels as much as possible and great attention should be paid to the relation with food intake the day preceding the blood sampling.

Conclusions

Pooled data from the above reviewed reports on the effect of homocysteine-lowering treatment in hyperhomocysteinemic vascular patients justify several conclusions. It should be possible to induce a reduction of about 40% of mildly elevated fasting homocysteine levels by the use of folic acid in a dose as low as 0.65 mg daily. A dose of 2.5 mg daily had the same effect, 5 mg daily resulted in a slightly larger decrease of about 50%, increasing the dose up to 10 mg daily had no extra effect. Supplementation of vitamin B_6 did not affect fasting hyperhomocysteinemia significantly. Vitamin B_{12} in an oral dose of 0.400 mg cyanocobalamin daily will have an only modest effect but it is advisable to add such dose to folic acid therapy more to avoid folic acid-refractoriness in case of vitamin B_{12} deficiency and to prohibit the development of neuropathy due to unrecognized perniciosa.

So, in summary, at present knowledge, the prescription of 0.65 mg folic acid plus 0.400 mg cyanocobalamin daily is required and sufficient to lower a mild fasting hyperhomocysteinemia for about 50%. The efficacy of even a lower dose of folic acid has not been explored so far. Indeed, a dose of folic acid of 0.65 mg is only 3 times higher than the recommended daily allowance (RDA 0.2 mg in most countries). However, to substitute vitamin suppletion in such low dose by dietary guidelines to increase folate intake from food, might be illusory because it would require a substantial change in ingrained dietary habits. Furthermore, it has been shown that dietary adjustment to guarantee the intake of at least 0.200 mg folic acid daily from the food was not able to maintain an acceptable folic acid blood level in subjects who tended to have low-normal concentrations[28].

Pooling the available data on the effect of various homocysteine-lowering regimen upon mildly elevated post-load homocysteine levels, learns that the use of 100 mg vitamin B_6 plus 5 mg folic acid daily after at least 6 weeks of treatment caused a decrease of these levels of about 50%. Vitamin B_6 as a single agent in doses from 100 to 250 mg daily resulted in a slightly lower reduction of about 40%. Folic acid as the sole therapy, however, has been studied only in 6 patients so far, but showed in these patients a 45% decrease. The efficacy of lower doses of vitamin B_6 and/or folic acid as single treatment or combined therapy has not been explored. Remarkably, only the combination of vitamin B_6 plus folic acid resulted in 90% or more of the treated patients in a complete normalization of the pathological post-load homocysteine levels, whereas the respective single treatments did much less, i.e. in about 50%. In the exceptional hyperhomocysteinemic patients who did not normalize after the use of vitamin B_6 plus folic acid, betaine in a dose of 6 g daily did always. In summary, at this state, in the treatment of post-load hyperhomocysteinemia it seems most preferable to

prescribe 100 mg vitamin B_6 plus 5 mg folic acid daily in view of the greatest efficacy of this combination and the highest rate of normalization of pathological homocysteine levels.

The above indicated recommendations for the treatment of fasting hyperhomocysteinemia are well in line with the design of a soon to start concerted action of several European countries in a placebo-controlled randomized dose-finding study[29]. The effect of treatment with folic acid in respective doses of 0.200 mg, 1 mg, and 5 mg, each in combination with 0.400 mg vitamin B_{12} will be compared with that of 0.400 mg vitamin B_{12} alone and with that of a placebo. In this design the apparently crucial role of folic acid as the universal homocysteine-lowering agent is well reflected. The still not completely clarified complexity of the aetiology of mild hyperhomocysteinemia either genetically or environmentally based, is much greater than originally thought. Notwithstanding that, the supplementation of folic acid seems to be the panacea in nearly all cases to lower the pathological homocysteine levels.

References

1. Sardharwalla IB, Fowler B, Robins AJ, Komrower GM. Detection of heterozygotes for homocystinuria. Arch Dis Child 1974;49:553-559.
2. Boers GHJ Hyperhomocysteinaemia: a newly recognized risk for vascular disease. Neth J Med 1994;45:34-41.
3 Boers GHJ, Smals AGH, Trijbels JMF, Fowler B, Bakkeren JAJM, Schoonderwaldt HC, Kleijer WJ, Kloppenborg PWC. Heterozygosity for homocystinuria in premature peripheral and cerebral occlusive arterial disease. N Engl J Med 1985;313:709-715.
4 Clarke R, Daly L, Robinson K, Naughten E, Cahalane S, Fowler B, Graham I. Hyperhomocysteinemia· an independent risk factor for vascular disease. N Engl J Med 1991;324:1149-1155.
5. Engbersen AMT, Franken DG, Boers GHJ, Stevens EMB, Trijbels JMF, Blom HJ. Thermolabile 5,10-methylenetetrahydrofolate reductase as a cause of mild hyperhomocysteinemia. Am J Hum Genet 1995;56:142-150.
6. Whitehead AS, Ward P, Tan S, Naughten E, Kraus JP, Sellar GC, McConnell DJ, Graha I, Gallagher PM. The molecular genetics of homocystinuria, hyperhomocysteinaemia, and premature vascular disease in Ireland. In: Mato JM, Caballero A (eds). Methionine metabolism: molecular mechanisms and clinical implications. Bouncopy Inc., Madrid 1994;79-83.
7 Kluijtmans LAJ, van den Heuvell LPWJ, Boers GHJ, Frosst P, Stevens EMB, Van Oost BA, Den Heijer M, Trijbels JMF, Rozen R, Blom HJ. Molecular genetic analysis in mild hyperhomocysteinemia: a common mutation in the methylenetetrahydrofolate reductase gene is a genetic risk factor for cardiovascular disease. Am J Hum Genet 1996;58:35-41.
8. Kang SS, Wong PWK, Susmano A, Sora J, Norusis M, Ruggie N. Thermolabile methylenetetrahydrofolate reductase: an inherited risk factor for coronary artery disease. Am J Hum Genet 1991;48·536-545.
9. Boers GHJ. Homocystinuria, a risk factor of premature vascular disease. Clinical Research Series, No 3. Foris Publications Dordrecht-Holland/Riverton-USA, 1986
10. Boers GHJ. The clinical usefulness of homocysteine determinations. In: Smith U, Eriksson S, Lindgarde F (eds). Genetic susceptibility to environmental factors, a challenge for public intervention. Almquist & Wiksell International Inc., Stockholm, 1988;35-42.
11. Franken DG, Boers GHJ, Blom HJ, Trijbels JMF, Kloppenborg PWC. Treatment of mild hyperhomocysteinemia in vascular disease patients. Arterioscl Thromb 1994;14:465-470.
12. Dordain G, Deffond D Neuropathies à la pyridoxine Therapie 1994,49:333-337
13. Brattstrom L, Israelsson B, Norrving B, Bergqvist D, Thorne J, Hultberg B, Hamfelt A Impaired homocysteine metabolism in early-onset cerebral and peripheral occlusive arterial disease. Atheroscler 1990;81:51-60.
14 Dudman NPB, Wilcken DEL, Wang J, Lynch JF, Macey D, Lundberg P. Disordered methionine/homocysteine metabolism in premature vascular disease. Its occurrence, cofactor therapy, and enzymology. Arterioscler Thromb 1993;13·1253-1260.
15. van den Berg M, Franken DG, Boers GHJ, Blom HJ, Jakobs C, Stehouwer CDA, Rauwerda JA. Combined vitamin B_6 plus folic acid therapy in young patients with arteriosclerosis and hyperhomocysteinemia. J Vasc Surg 1994;20·933-940.
16 Ubbink JB, Vermaak WJH, van der Merwe A, Becker PJ, Delport R, Potgieter HC. Vitamin requirements for the treatment of hyperhomocysteinemia in humans. J Nutr 1994;124.1927-1933.
17 Landgren F, Israelsson B, Hultberg B, Andersson A, Brattstrom L. Plasma homocysteine in acute myocardial infarction: homocysteine-lowering effect of folic acid. J Int Med 1995;237 381-388.

18. Brattström L, Israelsson B, Lindgärde F, Hultberg B. Higher total plasma homocysteine in vitamin B_{12} deficiency than in heterozygosity for homocystinuria due to cystathionine ß-synthase deficiency. Metabolism 1988;37:175-178.
19. Brattström LE, Israelsson B, Jeppsson JO, Hultberg BL. Folic acid, an innocuous means to reduce plasma homocysteine. Scand J clin Lab Invest 1988;48:215-221.
20. Allen RH, Stabler SP, Savage DG, Lindenbaum J. Diagnosis of cobalamin deficiency. 1: Usefulness of serum methylmalonic acid and total homocysteine concentrations. Am J Hematol 1990;34:90-98.
21. Franken DG, Boers GHJ, Blom HJ, Cruysberg JRM, Trijbels JMF, Hamel BCJ. Prevalence of familial mild hyperhomocysteinemia. Atheroscler 1996;125:71-80.
22. Ueland PM, Refsum H, Brattström L. Plasma homocysteine and cardiovascular disease. In: Francis RB (ed). Atherosclerotic cardiovascular disease, hemostasis, and endothelial function. Marcel Dekker Inc., New York, 1992;183-236.
23. Arnesen E, Refsum H, Bonaa KH, Ueland PM, Forde OH, Nordrehaug JE. Serum total homocysteine and coronary heart disease. Int J of Epidem 1995;24:704-709.
24. Stampfer MJ, Malinow MR, Willet WC, Newcomer LM, Upson B, Ullmann D, Tishler PV, Hennekens CH. A prospective study of plasma homocyst(e)ine and the risk of myocardial infarction in US physicians. JAMA 1992;268:877-881.
25. EC Comac Epidemiology Study 'Homocysteinaemia and vascular disease': annual report for 1989-1990. Leader: Graham I., Dublin. ECSC-EEC-EAEC Brussels-Luxembourg 1992.
26. Miller JW, Nadeau MR, Smith D, Selhub J. Vitamin B_6 deficiency versus folate deficiency: comparison of responses to methionine loading in rats. Am J Clin Nutr 1994;59:1033-1039.
27. Guttormsen AB, Schneede J, Fiskerstrand T, Ueland PM, Refsum HM. Plasma concentrations of homocysteine and other aminothiol compounds are related to food intake in healthy human subjects. J Nutr 1994;124:1934-1941.
28. Ubbink JB, van der Merwe A, Vermaak WJH, Delport R. Hyperhomocysteinemia and the reponse to vitamin supplementation. Clin Invest 1993;71:993-998.
29. EC Comac Epidemiology Study 'Homocysteine and vascular disease: from natural history to therapy', 1994. Leader: Graham I., Dublin.

TREATMENT OF MILD HYPERHOMOCYSTEINEMIA IN VASCULAR DISEASE PATIENTS

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Abstract

Mild hyperhomocysteinemia is recognized as a risk factor for premature arteriosclerotic disease. A few vitamins and other substances have been reported to reduce blood homocysteine levels, although, normalization of elevated blood homocysteine concentrations with any of these substances has hitherto not been reported. Therefore, we screened 421 patients suffering from premature peripheral or cerebral occlusive arterial disease by oral methionine loading tests for the presence of mild hyperhomocysteinemia. Thirty-three percent of patients with peripheral, and 20% of patients with cerebral occlusive arterial disease were identified with mild hyperhomocysteinemia, i.e. in 14% of the men, 34% of premenopausal and 26% of postmenopausal women. Mildly hyperhomocysteinemic patients were administered vitamin B_6, 250 mg daily. After six weeks, methionine loading tests were again assessed to evaluate the effect of treatment. Patients with non-normalized homocysteine concentrations were further treated with vitamin B_6, 250 mg daily, and/or folic acid, 5 mg daily, and/or betaine, 6 g daily, solely or in any combination. Vitamin B_6 treatment normalized the afterload homocysteine concentration in 56% of the treated patients, i.e. 71% in men, 45% in premenopausal, and 88% in postmenopausal women. Further treatment resulted in a normalization of homocysteine levels in 95% of the remaining cases. Thus, mild hyperhomocysteinemia, which is frequently encountered in patients with premature arteriosclerotic disease, can be reduced to normal in virtually all cases by safe and simple treatment with vitamin B_6, folic acid and betaine, substances each involved in methionine metabolism.

Introduction

Classic homocystinuria, due to homozygosity for cystathionine synthase (CS) deficiency, is characterized by severe accumulation of homocysteine in blood and tissues. The incidence of this hereditary dysfunction varies geographically and is estimated 1·200,000 worldwide[1]. Homocystinuria is generally considered to cause premature arteriosclerosis and thromboembolism. It is treated with high-dose administration of vitamin B_6, the active form, of which pyridoxal phosphate, functions as cofactor in the conversion of homocysteine into cystathionine (Figure 8.1). The marked homocysteine-lowering effect of vitamin B_6 is attributable to its stimulation of the residual activity of the cystathionine synthase enzyme[1 4]. Additionally, folic acid and betaine, both involved in the remethylation of homocysteine into methionine, can lower or even normalize the elevated homocysteine level in patients who respond poorly or not at all to vitamin B_6 treatment[1 3]. The incidence of vascular accidents is significantly reduced after initiating homocysteine-lowering treatment, revealing the clinically beneficial effect of such intervention in homozygous patients[4].

Mild hyperhomocysteinemia, with homocysteine concentrations equivalent to those as found in heterozygotes for CS deficiency, is characterized by mildly elevated blood homocysteine concentrations in the fasting state or after standardized methionine loading. However, intermediate CS deficiency is not the only possible genetic determinant of mild hyperhomocysteinemia. The occurrence of a mutant variant of methylenetetrahydrofolate reductase, characterized by 50% of normal activity and thermolability of the enzyme, has also been described recently in vascular patients with mild hyperhomocysteinemia[5]. Overall, in 9% to 42% of the patients under 50 years of age suffering from peripheral or cerebral occlusive arterial disease, myocardial infarction or thromboembolism, mild hyperhomocysteinemia has been observed and has come to be recognized as an independent risk factor for premature arteriosclerotic disease[6 24].

Routine screening for mild hyperhomocysteinemia among patients with signs of premature arteriosclerosis or thromboembolism at young age is recommendable if elevated homocysteine levels can be reduced by a safe and simple regimen that will produce a beneficial clinical effect. The homocysteine-lowering effect of vitamin B_6, folic acid or betaine has been observed in small groups of patients suffering from arteriosclerotic disease[12 25]. However, normalization of elevated homocysteine concentrations after methionine loading in large groups of hyperhomocysteinemic arteriosclerotic patients treated with high-dose administration of one or more of these compounds has not been reported. The present study not only included a large number of hyperhomocysteinemic patients, all suffering from premature peripheral or cerebral occlusive arterial disease, but, more importantly, also considered the homocysteine-normalizing effect of high-dose administration of vitamin B_6, folic acid, and betaine.

At the time of these studies the determination of the *free* homocysteine concentrations was routinely performed, which left the fasting levels unmanageably low. Therefore, only peak levels of homocysteine after methionine loading could be included. The introduction of the technique to measure total homocysteine, i.e. free plus protein bound, in our laboratory in 1991, after completion of this study, allowed a more sensitive determination of homocysteine blood levels in the fasting state. However, future studies will have to prove the

adequacy of sensitively and specificity of fasting total homocysteine levels for the establishment of a mildly hyperhomocysteinemic state in the individual vascular patient.

Materials and methods

Study population

During the period January 1980 through December 1990, 421 patients under 55 years of age with documented premature peripheral or cerebral occlusive arterial disease were screened for mild hyperhomocysteinemia by means of an oral methionine loading test. The diagnosis cerebral infarction was documented by cerebral computerized tomography scanning in all these patients. Patients with known risk factors such as diabetes (fasting plasma levels more than 7.3 mmol/L), hyperlipoproteinemia (fasting serum levels of cholesterol more than 6.5 mmol/L and triglycerides more than 2.00 mmol/L), and hypertension (systolic and diastolic blood pressure more than 150 mmHg and 95 mmHg, respectively) of non-

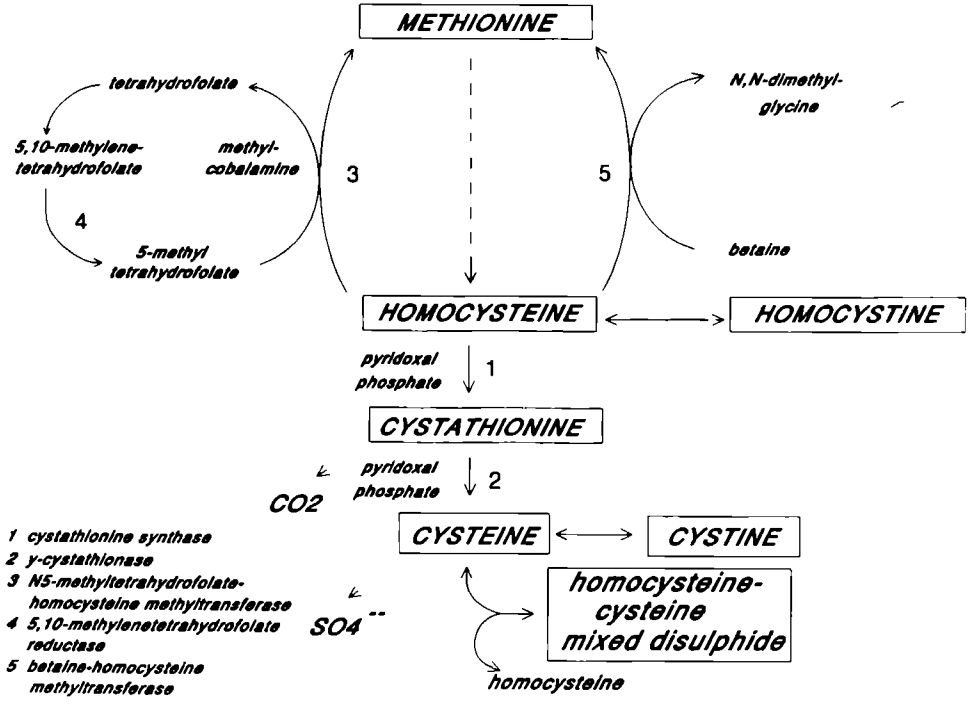

Figure 8.1 The metabolism of methionine. The transsulfuration of homocysteine into cystathionine (1, 2) and remethylation of homocysteine into methionine (3, 4, 5)

renovascular origin were excluded from the study. In all patients the blood levels of vitamin B_6 (normal range 28 - 107 nmol/L),B_{12} (160 -750 pmol/L), folic acid (5.5 - 40.0 nmol/L), creatinine, the liver enzymes (aspartate amino-transaminase and alanine amino-transaminase) were within the normal ranges.

Of the included patients, 131 (58 men and 73 women) had peripheral occlusive arterial disease, that had led to intermittent claudication or renovascular hypertension, and 290 (145 men and 145 women) had cerebral occlusive arterial disease, with a variety of persistent or transient neurologic signs (such as hemiplegia and aphasia). All patients originated from different kindreds.

The effect of homocysteine-lowering treatment could be evaluated retrospectively in 82 hyperhomocysteinemic patients, i.e. 21 men, 53 premenopausal, and 8 postmenopausal women with a mean age ± 1SD respectively 42 ± 8 years (range 16 - 55), 39 ± 6 years (range 24 - 50), and 50 ± 3 years (range 46 - 55).

In 63 controls a methionine loading test was performed to establish the normal range of post-load homocysteine levels. All control subjects were without vascular complications or any medication, and in all control subjects the vitamin B_6, B_{12} and folic acid concentrations were within the normal ranges. The control men (n = 20), premenopausal (n = 33) and postmenopausal (n = 10) women had a mean age ± 1SD of respectively 42 ± 11 years (range 22 - 55), 32 ± 10 years (range 16 -54), and 53 ± 3 years (range 45 - 55). Postmenopausality was questionairy been established by questionaaire (no menses for more than one year) and clinically ascertained by oestrogen level.

Methionine loading test and mild hyperhomocysteinemia

The methionine loading test was performed after an overnight fast. L-Methionine at a dose of 0.1g/kg body weight was administered orally in orange juice. During the next 8 hours the patient received a meal containing 14 mg of methionine. Four, six and eight hours after methionine loading blood samples were drawn. The blood samples were immediately centrifuged, the serum was deproteinized and stored at -20 °C until analysis. Free homocysteine concentrations were determined in all blood samples as homocystine and homocysteine-cysteine mixed disulphide concentrations by ion-exchange chromatography (Biotronik LC 2000 amino acid analyzer)[6,26,27]. Patients were considered to be hyperhomocysteinemic if their homocysteine peak level after methionine loading exceeded the mean peak level plus 2 standard deviations (SD) in the group of control subjects. Because of observed differences in mean homocysteine peak levels after methionine loading between control men, pre-, and postmenopausal women, the studied patients were categorized accordingly[6,26,27]. The peak in post-load homocysteine levels occurred in 87% of all performed tests at 6 hours, in 6% at 4 hours and in 7% at 8 hours after loading.

Protocol of homocysteine-lowering treatment

Patients identified as hyperhomocysteinemic received orally vitamin B_6 (pyridoxine hydrochloride), 250 mg daily. Six weeks later, with treatment continuing, a second methionine loading test was performed to assess the homocysteine-lowering effect. Because the aim of treatment was to normalize the elevated homocysteine level, we defined the reduction R by the formula:

$$R = \left(1 - \frac{L2 - Lc}{L1 - Lc}\right) \times 100\%$$

where L1 is homocysteine peak level after methionine loading before treatment, L2 is homocysteine peak level after methionine loading while on treatment, Lc is mean homocysteine peak level after methionine loading plus 2SD in control subjects.

We defined a reduction of L2 to Lc (or to a lower value) as 'normalization', a reduction of less than 60% as a 'poor or nonresponse' and a reduction of between 60% and 99% as an 'intermediate response'. This classification of the patient's response determined the mode of further treatment. In hyperhomocysteinemic patients in whom vitamin B_6 therapy failed to achieve normalization of the homocysteine peak level after methionine loading, the treatment was changed. In case of an intermediate response, vitamin B_6 therapy was continued, reinforced with folic acid, 5 mg daily, and in case of a poor or nonresponse vitamin B_6 was substituted by folic acid, 5 mg daily. If normalization of the hyperhomocysteinemia was shown by the methionine loading test performed 6 weeks after beginning the folic acid, no further change of treatment followed. In cases in which hyperhomocysteinemia persisted (intermediate or poor or nonresponse), betaine anhydrous (Sigma 2629), 6 g daily, was prescribed whereas folic acid was continued only if it achieved a 60% or greater reduction. The effect of betaine was also determined by a repeated methionine loading test 6 weeks after its start. In this regimen it was thus possible for the hyperhomocysteinemic patients to have a monotherapy of either vitamin B_6, folic acid or betaine, some combination of two of these, or even with the triple combination.

Statistics

The prevalence of mild hyperhomocysteinemia in the specified categories of patients and the respective effects of treatment were analyzed by a chi-square independence test. A probability value of 0.05 or less was considered statistically significant.

Results

Prevalence

Mild hyperhomocysteinemia was detected in 100 out of 421 vascular patients (24%), i.e. 29 out of 203 men (14%), 62 out of 183 premenopausal (34%) and 9 out of 35 postmenopausal women (26%) (Figure 8.2). The prevalence of mild hyperhomocysteinemia in premenopausal female patients was significantly higher than that in male patients ($p < 0.0001$), both in the peripheral subgroup ($p < 0.002$) and in the cerebral occlusive arterial disease subgroup ($p < 0.002$). The prevalence of mild hyperhomocysteinemia in all patients with peripheral occlusive arterial disease (33%) was significantly higher than that in patients with cerebral occlusive arterial disease (20%) ($p < 0.03$).

Effect of homocysteine-lowering treatment

After vitamin B_6 treatment for 6 weeks, in which the blood vitamin B_6 concentration in all 82 treated patients increased at least five fold compared to the pretreatment concentration, the homocysteine peak level after methionine loading

had normalized in 46 patients (56%), an intermediate response was found in 16 patients (20%), and in 20 patients (24%) the response was poor or absent (Figures 8.3 and 8.4). No significant difference in terms of normalization was detected between the subgroups of patients with peripheral and cerebral occlusive arterial disease (58% and 55%, respectively; $p > 0.8$). Vitamin B_6 treatment normalized the hyperhomocysteinemia in men significantly more frequently than in premenopausal women (71% versus 45%; $p < 0.05$). In terms of absolute reduction, the mean peak homocysteine concentration decreased after treatment from 30.5 ± 8.5 μmol/L to 17.7 ± 7.0 μmol/L (mean ± 1 SD) in male, from 28.5 ± 14.2 μmol/L

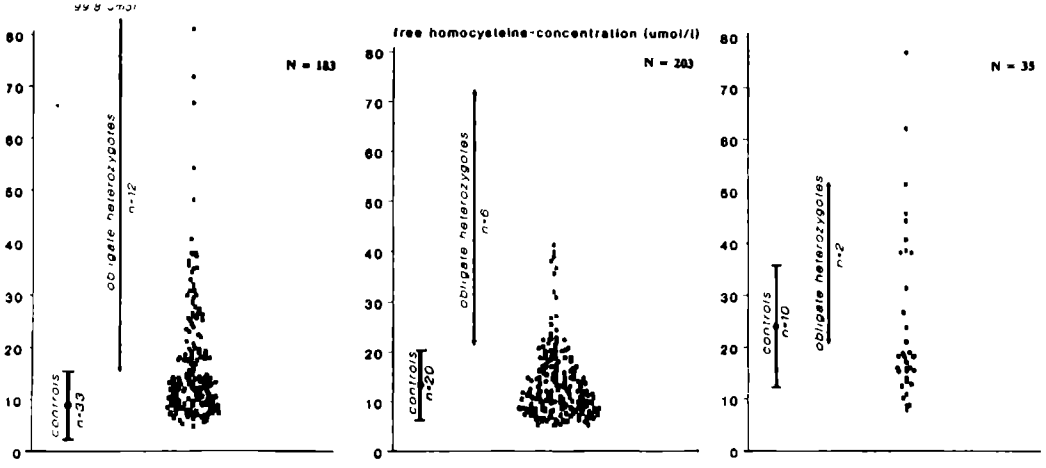

Figure 8.2. Individual free homocysteine levels after methionine loading (μmol/L) in 421 vascular male, premenopausal and postmenopausal female patients; the mean ± 2SD in control subjects and the range of obligate heterozygotes for cystathionine synthase deficient homocystinuria are indicated.

to 17.0 ± 7.2 μmol/L in premenopausal, and from 43.7 ± 5.5 μmol/L to 31.1 ± 22.7 μmol/L in postmenopausal patients.

Nine patients presenting an intermediate response and thirteen patients presenting a poor or nonresponse entered further the therapy as described in the section "Methods" (4 men, 17 premenopausal and 1 postmenopausal woman). All these patients yet normalized except one, who after continued treatment with vitamin B_6 and folic acid persisted in an intermediate response.

In conclusion, treatment of mild hyperhomocysteinemic patients suffering from peripheral or cerebral occlusive arterial disease resulted in 82% of treated patients in normalization of the homocysteine levels. Statistical analysis showed that if all patients in whom homocysteine levels after six weeks of vitamin B_6 treatment had not normalized and had received further treatment, normalization of the elevated homocysteine levels would have occurred in 98% of the patients.

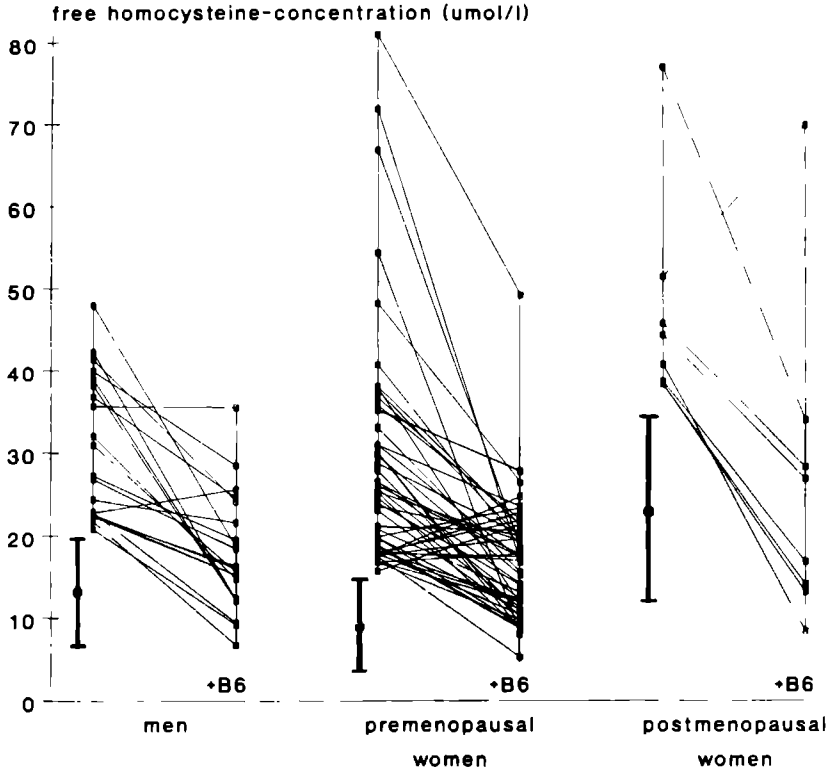

Figure 8.3. Response of the free homocysteine levels after methionine loading with 6 weeks of vitamin B_6 treatment, 250 mg daily, in 82 hyperhomocysteinemic vascular patients.

Discussion

The emphasis on hyperhomocysteinemia as a risk factor for premature arteriosclerosis and thromboembolism is based upon observations in patients suffering from classic homocystinuria[1 3]. From a collaborative study on more than 600 homozygotes for homocystinuria Mudd et al.[4] concluded that there was a chance of 50% for untreated patients to have a vascular accident before the age of 30 years. Homocysteine, a sulfhydryl amino acid formed by demethylation of methionine, is generally considered to be an atherogenic and thrombotic agent, although the pathogenic mechanism has not been clarified[1 28 34].

Mild hyperhomocysteinemia, with levels comparable to those in heterozygotes for homocystinuria, was detected by us previously in 28% of patients with premature peripheral or cerebral occlusive arterial disease without known risk factors such as diabetes, hyperlipoproteinemia, or non-renovascular hypertension[6] However, the number of patients, 25 in each group, was rather small. Since then, screening by a standardized methionine loading test of 421 such patients disclosed mild hyperhomocysteinemia in 33% of those with peripheral and in 20% of those with cerebral occlusive arterial disease. This is well in line both with our own earlier finding and with subsequent findings[6 21 35] Clarke et al.[17] using a methionine

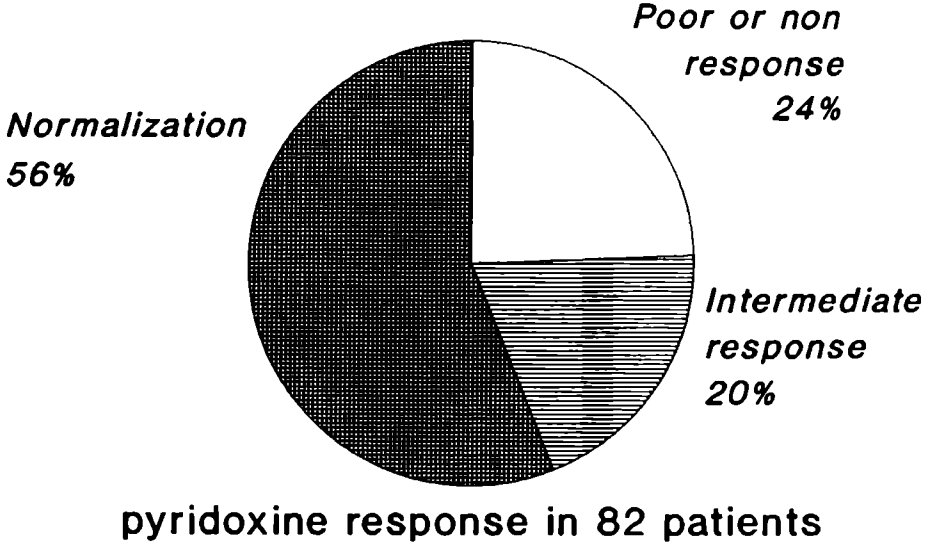

Figure 8 4 Individual free homocysteine levels after methionine loading before and after 6 weeks of vitamin B_6 treatment, 250 mg daily (n = 82).

loading test similar to ours, found mild hyperhomocysteinemia in up to 28% of 21 patients with peripheral and up to 42% of 38 patients with cerebral occlusive arterial disease. Patients with classic risk factors were not excluded in that study, but statistical analysis showed that mild hyperhomocysteinemia was an independent risk factor in addition to hypercholesterolemia, hypertension and smoking.

Remarkably, mild hyperhomocysteinemia was found to be significantly more prevalent among premenopausal female compared with male vascular patients. This finding confirms our earlier observation[6]. and was reconfirmed by Brattstrom et al.[12] after screening in 72 patients. We do not have a well-founded explanation for this sex difference in prevalence of mild hyperhomocysteinemia. We have speculated that lower levels of serum free homocysteine in premenopausal women compared with those in men may be a sign of more efficacious methionine handling, pointing to a defense mechanism that protects women during their reproductive years against vascular disease[27]. Failing such protection, as in case of mild hyperhomocysteinemia, young women seem more susceptible developing vascular disease than men The hormonal background also may act also protectively with respect to young women's conventional risk factors. To develop arteriosclerotic events they simply may need some extra and unconventional interference like hyperhomocysteinemia.

Now that mild hyperhomocysteinemia is generally recognized as one of the risk factors for the development of vascular disease, research into homocysteine-lowering treatment is needed. Homozygotes for homocystinuria, in whom serum free homocysteine levels in the fasting state may range as high as 200 to 300 μmol/L, respond in about 50% of cases to vitamin B_6 treatment in very high doses (up to 1 g daily)[4]. Although such treatment has been found to normalize the elevated homocysteine levels in the fasting state, it does not enhance the patient's capacity to efficiently handle a major methionine load[2] Treatment with folic acid, betaine or dietary methionine restriction results in a reduction of homocysteine levels in the majority of the patients who respond poorly or not at all to vitamin B_6 treatment[3 36] A significantly reduced number of initial vascular events following reduction of pathologically high homocysteine levels has been shown retrospectively in a large group of homozygotes for homocystinuria[4].

Present study retrospectively reviewed normalization of mildly elevated homocysteine levels after methionine intake in a large group of vascular patients with vitamin B_6, folic acid and betaine treatment. Brattstrom et al [12] report a 26% reduction of mildly elevated homocysteine levels after methionine loading in 20 vascular patients after two weeks of vitamin B_6 treatment, 240 mg daily. After two more weeks of treatment with vitamin B_6, 240 mg daily, plus folic acid, 10 mg daily, a total reduction of elevated homocysteine levels of 39% was achieved. However, normalization of methionine handling by such treatment was not mentioned. In the present study, vitamin B_6 was treatment of first choice, and normalization of the homocysteine peak level after methionine loading was achieved in 56% of the patients. Normalization was observed considerably less frequently in premenopausal patients than in male and postmenopausal patients, 45,% 71% and 88%, respectively. From this observation one might hypothesize that a different mechanism for hyperhomocysteinemia is responsible in young females Vitamin B_6, folic acid and betaine solely or in any combination led in

virtually all treated patients to normalization of their overresponse by homocysteine to methionine loading. In view of these findings we have recently treated 21 newly detected hyperhomocysteinemic patients with vitamin B_6, 250 mg daily, combined with folic acid, 5 mg daily, and have found all of them to present normal responses to methionine loading within 6 weeks (data not shown).

In 18 patients from the present study in whom normalization of the homocysteine levels had been achieved with vitamin B_6, folic acid or betaine solely or in any combination, a methionine loading test performed after one year of continued treatment showed persistence of the normalized homocysteine levels (data not shown).

Enzyme activity determinations were not been performed on a routine base in the studied patients with mild hyperhomocysteinemia. In about half of them the vitamin B_6 treatment corrected the homocysteine peak level, which suggests that these patients are heterozygotes for CS deficiency. The remaining patients in whom homocysteine handling was not normalized by vitamin B_6 might be affected by either a vitamin B_6-nonresponding CS mutant in heterozygous form or by a defect in homocysteine remethylation. The latter may be the thermolabile form of 5,10-methylenetetrahydrofolate reductase (Figure 8.1), reported by Kang et al.[5]. This defect occurred in 17% of patients with coronary artery disease, although it did not automatically lead to mild hyperhomocysteinemia in the fasting state in the patients.

Vitamin B_6 at doses from 200 mg up to 6 g has sporadically been considered as a cause of sensory neuropathy[37,38]. Remarkably, these side effects were not observed in patients with homocystinuria who received long-term treatment (up to 24 years) with doses up to 750 mg[3,39]. Folic acid and betaine at the dosages prescribed by us being 5 mg and 6 g daily, respectively, are not reported to produce side effects[36,40,41].

The present study proved that normalization of mild hyperhomocysteinemia is attainable in virtually all patients by safe and simple treatment with vitamin B_6, folic acid and betaine. Further dose-response studies will disclose the lowest required dosage of these substances.

A decreased number of vascular events in arteriosclerotic patients with mild hyperhomocysteinemia due to homocysteine-lowering treatment has not been demonstrated so far. We are presently conducting a placebo-controlled intervention study that may provide a clinical justification for screening in prematurely arteriosclerotic patients.

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References

1. Mudd SH, Levy HL, Skovby F. Disorders of transsulfuration. In: Scriver CR, Beaudet AL, Sly WS, Valle D (eds). The metabolic basis of inherited disease. McGraw-Hill Inc., New York, 1989;693-734.
2. Boers GHJ, Smals AGH, Drayer JIM, Trijbels JMF, Leermakers AI, Kloppenborg PWC. Pyridoxine treatment does not prevent homocystinemia after methionine loading in adult homocystinuria patients. Metabolism 1983;32:390-397.
3. Boers GHJ. Homocystinuria, a risk factor of premature vascular disease. Clinical Research Series no 3. Dordrecht-Holland/Riverton-USA, Foris Publications, 1986.
4. Mudd SH, Skovby F, Levy HL, Pettigrew KD, Wilcken B, Pyeritz RE, Andria G, Boers GHJ, Bromberg IL, Cerone R, Fowler B, Gröbe H, Schmidt H, Schweitzer L. The natural history of homocystinuria due to cystathionine ß-synthase deficiency. Am J Hum Genet 1985;37:1-31.
5. Kang S-S, Wong PWK, Susmano A, Norusis M, Ruggie N. Thermolabile methyltetrahydrofolate reductase: an inherited risk factor for coronary artery disease. Am J Hum Genet 1991;48:536-545.
6. Boers GHJ, Smals AGH, Trijbels JMF, Fowler B, Bakkeren JAJM, Schoonderwaldt HC, Kleijer WJ, Kloppenborg PWC. Heterozygosity for homocystinuria in premature peripheral and cerebral occlusive arterial disease. N Engl J Med 1985;313:709-715.
7. Brattstrom LE, Hardebo JE, Hultberg BL. Moderate homocysteinemia - a possible risk factor for arteriosclerotic cerebrovascular disease. Stroke 1984;15:1012-1016.
8. Murphy-Chutorian DR, Wexman MP, Grieco AJ, Heiniger JA, Glassman E, Gaull GE, Steven KC, Feit F, Wexman K, Fox AC. Methionine intolerance: a possible risk factor for coronary artery disease. J Am Coll Cardiol 1985;6:725-730.
9. Kang S-S, Wong PWK, Cook HY, Norusis M, Messner JV. Protein-bound homocyst(e)ine. A possible risk factor for coronary artery disease. J Clin Invest 1986;77:1482-1486.
10. Israelsson B, Brattstrom LE, Hultberg BL. Homocysteine and myocardial infarction. Atherosclerosis 1988;71:227-233.
11. Malinow MR, Kang SS, Taylor LM, Wong PWK, Coull B, Inahara T, Mukerjee D, Sexton G, Upson B. Prevalence of hyperhomocyst(e)inemia in patients with peripheral arterial occlusive disease. Circulation 1989,79:1180-1188.
12. Brattstrom L, Israelsson B, Norrving B, Bergqvist D, Thorne J, Hultberg B, Hamfelt A. Impaired homocysteine metabolism in early-onset cerebral and peripheral occlusive arterial disease. Effects of pyridoxine and folic acid treatment. Atherosclerosis 1990;81:51-60.
13. Malinow MR, Sexton G, Averbuch M, Grossman M, Wilson D, Upson B. Homocyst(e)inemia in daily practice: levels in coronary artery disease. Cor Art Dis 1990;1:215-220.
14. Coull BM, Malinow MR, Beamer N, Sexton G, Nordt F, deGarmo P. Elevated plasma homocyst(e)ine concentration as a possible independent risk factor for stroke. Stroke 1990;21:572-576.
15. Genest JJ, McNamara JR, Upson B, Salem DN, Ordovas JM, Schaefer EJ, Malinow MR. Prevalence of familial hyperhomocyst(e)inemia in men with premature coronary artery disease. Arteriosclerosis and Thrombosis 1991;11·1129-1136.
16. Ubbink JB, Vermaak WJK, Bennett JM, Becker PJ, Staden DA, Bissbort S. The prevalence of homocysteinemia and hypercholesterolemia in angiographically defined coronary heart disease. Klin Wochenschr 1991;69:527-534.
17. Clarke R, Daly L, Robinson K, Naughten E, Cahalane S, Fowler B, Graham I. Hyperhomocysteinemia: an independent risk factor for vascular disease. N

Engl J Med 1991;324:1149-1155.
18. Taylor LM, DeFrang RD, Harris EJ, Porter JM. The association of elevated plasma homocyst(e)ine with progression of symptomatic peripheral arterial disease. J Vasc Surg 1991;13:128-136.
19. Brattström L, Tengborn L, Lagerstedt C, Israelsson B, Hultberg B. Plasma homocysteine in venous thromboembolism. Haemostasis 1991;21:51-57.
20. Bienvenu T, Ankri A, Chadefaux B, Kamoun P. Dosage de L'homocystéine plasmatique dans l'exploration des thromboses du sujet jeune. Presse Méd 1991;21:985-988.
21. Mölgaard J, Malinow MR, Lassvik C, Holm A-C, Upson B, Olsson AG. Hyperhomocyst(e)inaemia: an independent risk factor for intermittent claudication. J of Int Med 1992;231:273-279.
22. Brattström L, Lindgren A. Hyperhomocysteinemia as a risk factor for stroke. Neurol Res 1992;14:81-84.
23. Kang S-S, Wong PWK, Malinow MR. Hyperhomocysteinemia as a risk factor for occlusive vascular disease. Annu Rev Nutr 1992;12:279-298.
24. Ueland PM, Refsum H, Brattström L. Plasma homocysteine and cardiovascular disease. In: Francis RBF (ed). Atherosclerotic cardiovascular disease, hemostasis, and endothelial function. Marcel Dekker Inc., New York, 1992;183-236.
25. Boers GHJ. The clinical usefulness of homocysteine determinations. In Smith U, Eriksson S, Lingarde F (eds). Genetic susceptibility to environmental factors - a challenge for public intervention. Almqvist, Wiksell International Inc., Stockholm, 1988;35-42.
26. Boers GHJ, Fowler B, Smals AGH, Trijbels JMF, Leermakers AI, Kleijer WJ, Kloppenborg PWC. Improved identification of heterozygotes for homocystinuria due to cystathionine synthase deficiency by the combination of methionine loading and enzyme determination in cultured fibroblasts. Hum Genet 1985;69:164-169.
27. Boers GHJ, Smals AGH, Trijbels JMF, Leermakers AI, Kloppenborg PWC. Unique efficiency of methionine metabolism in premenopausal women may protect against vascular disease in the reproductive years. J Clin Invest 1983;72:1971-1976.
28. Starkebaum G, Harlan JM. Endothelial cell injury due to copper-catalyzed hydrogen peroxide generation from homocysteine. J Clin Invest 1986;77:1370-1376.
29. Parthasarathy S. Oxidation of low density lipoprotein by thiol compounds leads to its recognition by the acetyl LDL receptor. Biochem Biophys Acta 1987;917:337-340.
30. Dudman NPB, Hicks C, Wang J, Wilcken DEL. Human arterial endothelial cell detachment in vitro: its promotion by homocysteine and cysteine. Atherosclerosis 1991;91:77-83.
31. Blom HJ, Engelen DPE, Boers GHJ, Stadhouders AM, Sengers RCA, de Abreu R, te Poele-Pothoff MTWB, Trijbels JMF. Lipid peroxidation in homocysteinaemia. J Inher Metab Dis 1992;15:419-422.
32. Dudman NPB, Wilcken DEL, Stocker R. Circulating lipid hydroperoxide levels in human hyperhomocysteinemia - Relevance to development of arteriosclerosis. Arteriosclerosis and Thrombosis 1993;13:512-516.
33. Lentz SR, Sadler JE. Inhibition of thrombomodulin surface expression and protein C activation by the thrombogenic agent homocysteine. J Clin Invest 1991;88:1906-1914.
34. Stamler JS, Osborne JA, Jaraki O, Rabbani LE, Mullins M, Singel D, Loscalzo J. Adverse vascular effects of homocysteine are modulated by endothelium-derived relaxing factor and related oxides of nitrogen. J Clin Invest 1993;91:308-318.
35. Boers GHJ, Schoonderwaldt HC, Schulte BPM, Trijbels JMF, Smals AGH, Kloppenborg PWC. Heterozygosity for homocystinuria: a risk factor of

occlusive cerebrovascular disease? Clin Genet 1983;24:300-301.
36. Wilcken DEL, Wilcken B, Dudman NPB, Tyrell PA. Homocystinuria - the effects of betaine in the treatment of patients not responsive to pyridoxine. N Engl J Med 1983;309:448-453.
37. Parry GJ, Bredensen DE. Sensory neuropathy with low dose pyridoxine. Neurology 1985;35:1466-1468.
38. Schaumberg H, Kaplan J, Windebanke A, Vick N, Rasmus S, Pleasure D, Brown MJ. Sensory neuropathy from pyridoxine abuse. N Engl J Med 1983;309:445-448.
39. Mpofu C, Alani SM, Whitehouse C, Fowler B, Wraith JE. No sensory neuropathy during pyridoxine treatment in homocystinuria. Arch Dis Child 1991;66:1081-1082.
40. Brody T, Shane B, Stokstad ELR. Folic acid. In: Machlin LJ (ed). Handbook of vitamins, nutritional, biochemical, and clinical aspects. Marcel Dekker Inc., New York and Basel, 1984;459-496.
41. Friedrich W. Folic acid and unconjugated pteridines. In: Friedrich W (ed). Vitamins. Walter de Gruyter Inc., New York, 1988;619-752.

COMBINED VITAMIN B_6 PLUS FOLIC ACID THERAPY IN YOUNG PATIENTS WITH ARTERIOSCLEROSIS AND HYPERHOMOCYSTEINEMIA

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Abstract

Hyperhomocysteinemia is associated with arteriosclerotic and thromboembolic events. The homocysteine-lowering effect of combined treatment with vitamin B_6 plus folic acid has never been explored in a large group of vascular patients. Therefore, we studied the effects of at least 6 weeks treatment with these vitamins in 72 patients with cardiovascular disease and mild hyperhomocysteinemia (defined as an increase of the plasma homocysteine level after methionine loading >97.5 percentile of age-matched controls but < 200 μmol/L).

The existence of mild hyperhomocysteinemia was investigated in 309 consecutive patients under 50 years of age suffering from peripheral arterial occlusive disease, cerebral arterial occlusive disease, or coronary artery occlusive disease. All patients with an abnormal loading test were treated with vitamin B_6, 250 mg daily, plus folic acid, 5 mg daily. After 6 weeks of treatment a second methionine loading test was performed to assess the homocysteine-lowering effect.

Mild hyperhomocysteinemia was detected in 72 patients (23%), 33 (46%) of whom also had hyperhomocysteinemia when fasting. Treatment with vitamin B_6 plus folic acid normalized the post-load plasma homocysteine concentration in 66 of the 72 patients (92%), whereas fasting hyperhomocysteinemia was normalized in 30 of 33 (91%) patients. In 6 patients therapy failed to achieve normalization of the post-load homocysteine levels. In 3 of these patients, the same treatment was continued for a further 6 weeks and in the remaining 3 patients betaine was added to the treatment regimen. After 6 weeks of additional treatment all these 6 patients had normal post-load plasma homocysteine concentrations.

The prevalence of mild hyperhomocysteinemia in young patients with arterial occlusive disease is high. Simple and inexpensive therapy with vitamin B_6 plus folic acid will normalize homocysteine metabolism, as assessed by the homocysteine plasma level after methionine loading, in virtually all these patients.

Introduction

Homocystinuria in its classic form is an autosomally inherited disease characterized by very high plasma levels (>200 μmol/L) of the thiol-containing amino acid homocysteine. Patients may have ectopia lentis, skeletal abnormalities, mental retardation, Marfanoid features, thrombosis and premature arteriosclerosis[1]. These patients with severe hyperhomocysteinemia can be successfully treated by administration of high doses of vitamin B_6, which lowers plasma concentrations of homocysteine by stimulating the residual cystathionine synthase (CS) activity, which converts homocysteine to cystathionine (Figure 9.1)[1,2]. In case of a poor response to vitamin B_6 therapy, addition of folic acid or betaine may result in a dramatic decrease of the homocysteine levels because of enhanced remethylation of homocysteine to methionine[3,4]. Therapy with these cofactors or cosubstrates of homocysteine metabolism reduces the risk of cardiovascular events in patients with classic homocystinuria[4].

Recent studies indicate a relationship between mild hyperhomocysteinemia (mHH) and arterial occlusive disease[5-8]. The impairment of homocysteine metabolism in cases of mHH may be caused by deficient activity of cystathionine synthase (CS), methylenetetrahydrofolate reductase (MTHFR) or other still unknown enzyme deficiencies (Figure 9.1),[7,9,10] or by environmental factors such as deficiencies of the vitamins B_6, B_{12} and folic acid[5,6]. In patients under 50 years of age diagnosed with peripheral arterial occlusive disease, cerebral arterial occlusive disease or coronary artery occlusive disease, an excessive homocysteine concentration after oral methionine loading has been observed with a prevalence varying from 8% to 42%[5,7,8,11]. As in classic, severe homocystinuria, treatment with cofactors or cosubstrates of homocysteine metabolism may lower the plasma homocysteine concentration in patients with arteriosclerosis and mHH[7,12]. In a group of 20 vascular patients with mHH Brattström et al.[12] reported a mean reduction of the post-load homocysteine concentration after treatment with vitamin B_6 of 26%, whereas the mean fasting level was unchanged. When vitamin B_6 was combined with folic acid, the mean reduction was 39% for post-load levels and 53% for fasting levels[12]. However, the relatively small number of patients studied by Brattström et al.[12] precluded a precise assessment of the efficacy of treatment with vitamin B_6 plus folic acid, both in terms of the reduction of plasma homocysteine levels after methionine loading and in terms of the percentage of patients in whom the methionine loading test result normalized.

We, therefore, investigated the prevalence of mHH (as defined by homocysteine levels after methionine loading) in 309 consecutive patients younger than 50 years of age with symptomatic arterial occlusive disease. Seventy-two patients had pathological tests (23%). In these patients, we studied the effects of treatment with vitamin B_6 plus folic acid on both fasting and post-load homocysteine concentrations.

Patients and methods

Patients

From March 1991, until February 1993, we studied 309 patients with symptomatic vascular disease (198 men and 111 women under 50 years of age) consecutively admitted to a large secondary referral center. All these patients had symptomatic peripheral arterial occlusive disease, cerebral arterial occlusive

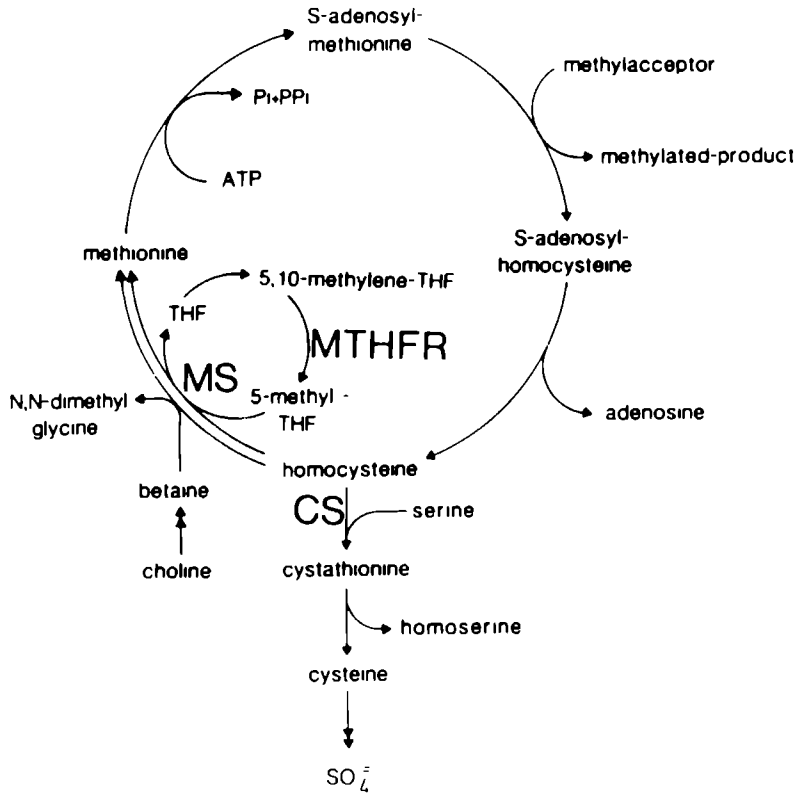

Figure 9.1. Methionine/homocysteine metabolism. CS (cystathionine synthase), MTHFR (5,10-methylenetetrahydrofolate reductase), MS (methionine synthase), THF (tetrahydrofolate).

disease, or coronary artery occlusive disease. Peripheral arterial occlusive disease was defined by the presence of intermittent claudication confirmed by ankle/arm indexes <0.9 or a decrease of >0.15 of the index on treadmill testing, or ischemic rest pain, gangrenous ulcers, or amputation for ischemia. Cerebral arterial occlusive disease was defined by the presence of symptomatic cerebral vascular disease (ischemic stroke or transient ischemic attack, World Health Organization [WHO] clinical definitions) and, in the case of stroke, was confirmed by computed tomography. Coronary artery occlusive disease was defined as the presence of myocardial infarction (WHO clinical definition plus new Q waves on electrocardiography or diagnostic enzyme changes). This restrictive definition of

coronary artery disease was used because we wanted to avoid the possibility of misclassification in view of the somewhat uncertain relation between hyperhomocysteinemia and coronary artery disease[5-8]. All these patients had normal levels of vitamin B_6 (>17 nmol/L; fluorescence high performance liquid chromatography = HPLC), vitamin B_{12} (>120 pmol/L; radioassay, Becton Dickinson, Erembodegem-Aalst, Belgium) and folic acid (>3.4 nmol/L; radioassay, Becton Dickinson) and no signs of renal failure (serum creatinine <120 μmol/L) or liver dysfunction (serum aspartate aminotransaminase and alanine aminotransaminase <30 U/L and absence of physical signs). Of these patients, 143 (46%) had peripheral disease, 86 (28%) had cerebral disease and 80 (26%) had a myocardial infarction. We recorded risk factors for occlusive arterial disease (diabetes mellitus [WHO criteria], hyperlipoproteinemia [total cholesterol >6.2 mmol/L or triglycerides >2 mmol/L], current smoking habits and hypertension [diastolic blood pressure >90 mmHg or systolic blood pressure >140 mmHg (measured after 15 minutes of rest in the supine position without altering antihypertensive regimens) or taking antihypertensive drugs]).

Methods

After an overnight fast, blood was drawn for measurement of serum total cholesterol and triglycerides (measured enzymatically), creatinine (modified Jaffé reaction), and glucose (glucose oxidase method). For technical reasons we did not measure high-density lipoprotein cholesterol. The presence of mHH was studied by an oral methionine loading test. An ethylenediamine tetraacetic acid (EDTA) plasma sample for determination of the fasting homocysteine concentration was drawn at 8 AM. After it was centrifuged for 10 minutes at 1800 G, plasma was stored at -30 °C until analysis. Next, an oral dose of L-methionine (0.1 gram/kg body weight) was administered in orange juice. During the test the patients ate a standardized methionine-poor breakfast and lunch (containing 14 mg methionine/gram protein). Six hours after the methionine load a second EDTA-plasma sample was drawn for determination of the post-load homocysteine concentration. All measurements were performed within one week.

Total (free plus protein bound) homocysteine concentrations (disulphide homocysteine plus mixed disulphides) were measured by use of tri-n-butylphosphine as the reducing agent and ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate as the fluorochromophore, followed by HPLC with fluorescence detection[13]. Intraassay and interassay coefficient of variation for total homocysteine were 2.1% and 5.1%, respectively.

Patients were considered to have mHH if homocysteine concentrations measured 6 hours after methionine loading exceeded the 97.5 percentile in healthy volunteers and were lower than (arbitrarily) 200 μmol/L. Healthy volunteers (mean age (SD) for men and women, 36.9 (5.7) and 42.3 (9.5), respectively) were recruited from the hospital staff and had vitamin B_6, B_{12}, and folic acid concentrations within the normal ranges. In healthy men (n = 23), the mean post-load homocysteine concentration (2.5-97.5 percentile) was 37 (25-54) μmol/L, in healthy premenopausal women (n = 41) it was 31 (18-51) μmol/L, and in healthy postmenopausal women (n = 27) it was 43 (25-69) μmol/L. Mean fasting homocysteine levels in these control groups were 12 (8-18), 10 (6-15), and 11 (6-19) μmol/L, respectively.

Therapy

Patients with mHH were treated with vitamin B_6 (pyridoxine), 250 mg daily, in combination with folic acid, 5 mg daily. After 6 weeks of treatment, a second methionine loading test was performed to assess the homocysteine-lowering effect. In those patients in whom normalization of the post-load homocysteine concentration was not achieved, a third methionine loading test was performed after another 6 weeks of further treatment.

Data are given as mean (95%-confidence interval [CI]), or as mean (SD), unless otherwise indicated. Significance was tested by the two-tailed Student t-test and the Chi-square independence test. Correlations were studied with Pearson's or Spearman's tests, as appropriate. The p values <0.05 were considered significant.

Results

We observed a normal post-load homocysteine level in 237 of the 309 patients with arteriosclerotic arterial occlusive disease (77%), whereas fasting levels were normal in 266 patients (86%). In patients with normal post-load levels ($n = 237$), the mean homocysteine concentrations were 35.5 (8.3), 33.9 (11.1) and 39 (9.5) μmol/L for men, premenopausal women and postmenopausal women, respectively. In patients with normal fasting levels ($n = 266$), the mean fasting homocysteine levels were 10.8 (2.9), 9.3 (2.7), and 10.1 (3.0) μmol/L.

Prevalence

Mild post-load hyperhomocysteinemia was present in 72 of the 309 patients (23%; 95%-confidence interval [CI], 19-28), i.e., 36 of 143 patients with peripheral disease (25%; CI 18-32), 24 of 86 patients with cerebral disease (28%; CI 19-39) and 12 of 80 patients with coronary disease (15%; CI 8-25). The prevalence of other risk factors (hyperlipoproteinemia, smoking habits, diabetes mellitus and hypertension) was similar in the patients with and without mHH (Table 9.1). However, in patients with hyperhomocysteinemia, the percentage of men was significantly lower than in patients without hyperhomocysteinemia (43% versus 64%, $p < 0.05$). Fasting hyperhomocysteinemia was observed in 33 of the 72 patients with post-load hyperhomocysteinemia (46%). Of these 33 patients, 17 patients had peripheral vascular disease, 10 patients had cerebral artery disease, and 6 patients had coronary artery disease, a distribution that was not significantly different from the distribution observed in the 72 patients with post-load hyperhomocysteinemia. Patients with ($n = 33$) and without ($n = 39$) fasting hyperhomocysteinemia were not significantly different with regard to the prevalence of other risk factors (hyperlipoproteinemia, 30% versus 28%; hypertension, 24% vs 21%; smokers, 75% vs 82%; diabetes, 3% vs 3%). The sex ratio was also similar (47% vs 36% men).

Effects of therapy.

Treatment with vitamin B_6 plus folic acid had no effect on the fasting glucose, cholesterol, triglyceride and creatinine levels (Table 9.2). After 6 weeks of treatment, the mean blood concentrations of both vitamin B_6 and folic acid had increased at least fivefold compared to the pretreatment concentration. After 6 weeks of treatment 66 of the 72 patients (92%; CI 83-97) showed normal plasma

Table 9 1. Prevalence of risk factor in patients with and without hyperhomocysteinemia.

Risk factor	Hyperhomocysteinemia present	absent	P Value*
Mean age(yr)	43.8(3.2)	44.6(2.1)	NS
Male sex (%)	42 (30-54)	64 (58-70)	< 0.05 **
Hyperlipoproteinemia (%)	30 (19-41)	24 (19-30)	NS
Smoking (%)	79 (68-88)	83 (78-88)	NS
Diabetes (%)	3 (0-10)	4 (2-7)	NS
Hypertension (%)	23 (13-34)	19 (14-24)	NS

NS = Not significant; *Chi-square analysis; **In the total group the ratio male:female was 198:111. Numbers in parenthese indicate 95% CI.

Table 9.2. Effect on serum levels of glucose, cholesterol, triglycerides, and creatinine of 6 weeks of treatment with vitamin B_6 plus folic acid in patients with mild hyperhomocysteinemia.

Value*	Before treatment (n = 72)	After treatment (n = 72)	P
Glucose	5.0 (0.4)	5.0 (1.2)	NS
Cholesterol	6.0 (1.1)	6.1 (1.1)	NS
Triglycerides	1.8 (0.9)	1.8 (1.4)	NS
Creatinine	83 (14)	84 (15)	NS

NS = Not significant ($p > 0.05$); *Student t-test; Values expressed as mean (SD).

homocysteine concentrations after methionine loading, whereas fasting homocysteine levels normalized in 30 of the 33 patients (91%; CI 76-98). In 6 patients (1 man, 5 premenopausal women) the post-load homocysteine concentration had not normalized after 6 weeks of therapy. Three patients (1 man, 2 premenopausal women) continued their therapy for an additional 6 weeks, which resulted in a normalization of the post-load plasma homocysteine concentration. In the 3 remaining patients (3 premenopausal women) betaine anhydrous (Sigma 2629; Sigma Chemical Co., St. Louis, Mo), 6 g daily, was added to the treatment regimen for 6 weeks. In these 3 patients the post-load homocysteine levels also normalized.

Treatment resulted in a mean reduction of the post-load plasma homocysteine concentration of 48% (CI 35-57) in all patients after 6 to 12 weeks. The mean reduction of the fasting homocysteine concentration was 51% (CI 32-64) after 6 weeks. The mean decrease in the post-load homocysteine concentration after 6 to 12 weeks of therapy was similar in men (46%; CI 25-86), premenopausal women (48%, CI 35-58) and postmenopausal women (55%; CI 39-74; Table 9 3, Figure 9.2). The mean decrease in fasting homocysteine concentrations was 56% (CI 40-69) in men, 46% (CI 31-59) in premenopausal women, and 51% (CI 1-94) in postmenopausal women, respectively. The responses to therapy were not significantly different among the groups of men and premenopausal or

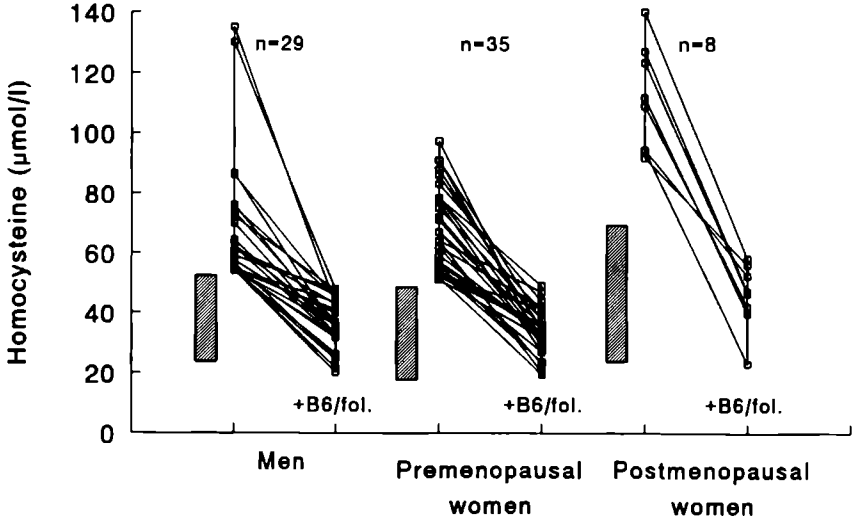

Figure 9.2. Post-load homocysteine levels before and after 6 to 12 weeks of treatment with vitamin B_6 plus folic acid in hyperhomocysteinemic men, premenopausal women and postmenopausal women with mild hyperhomocysteinemia. The shaded rectangles indicate normal ranges (2.5 to 97.5 percentile).

Table 9.3. Mean post-load homocysteine concentrations in 72 patients with hyperhomocysteinemia before and after treatment with vitamin B_6 plus folic acid.

Group	Number of patients	Before treatment		After treatment†	
		fasting	post-load	fasting	post-load
All patients	72	19 (13)	71 (21)	9 (3)	37 (12)§
Men	29	23 (15)	69 (21)	10 (3)	37 (10)§
Premenopausal women	35	15 (4)	64 (15)	8 (4)	33 (12)§
Postmenopausal women	8	20 (12)	109 (14)	10 (2)	49 (15)‡

† In all groups, there were significant differences between fasting homocysteine concentrations (before versus after treatment) and between post-load concentrations (before versus after). § = $p < 0.001$, and ‡ = $p < 0.02$ (Student's t-test). Values expressed as mean (SD) µmol/L.

postmenopausal women, or between the fasting and post-load levels Similarly, the response to treatment did not differ significantly according to the location of vascular disease. Thus, the mean decreases in fasting and post-load homocysteine concentrations were 53% (CI 23-84) and 46% (CI 30-62) in patients with peripheral occlusive disease, 48%(CI 17-78) and 49% (CI 30-65) in patients with cerebral occlusive disease, and 49% (CI 11-86) and 52% (CI 33-69) in patients with myocardial infarction, respectively. Fasting homocysteine levels, either before or after treatment, were not related to serum creatinine ($r = 0.19$, $p = 0.21$ and $r = -0\ 001$, $p = 0.9$, respectively). Post-load homocysteine levels were related to serum creatinine before ($r = 0.36$, $p = 0.02$) but not after treatment ($r = -0\ 04$, $p = 0.6$)

Discussion

Pathogenesis

Increased levels of plasma homocysteine are generally accepted as one of the causes leading to arteriosclerosis and thromboembolism[1]. In vitro, homocysteine induces endothelial cell injury and affects endothelial cell function[14-16] Homocysteine is belived to damage endothelial cells by several mechanisms, such as generation of hydrogen peroxide and depletion of nitric oxide-mediated detoxification of homocysteine[17,18]. In patients with classic homocystinuria, the high homocysteine concentrations are most commonly caused by a deficiency of CS activity[1,2] In contrast, mHH may have both environmental and genetic causes. First, elevated homocysteine levels have been observed in patients with kidney failure (through unknown mechanisms) and in patients with deficiencies of cofactors or cosubstrates of methionine metabolism (folic acid, vitamin B_{12}, or vitamin B_6 [Figure 9 1])[5]. Second, mildly elevated homocysteine levels may be genetically determined[5], although the primary defect in such patients has not been elucidated Apart from CS deficiency, a decreased activity of 5,10-methylenetetrahydrofolate reductase or other unknown enzyme deficiencies have been suggested[7,10]

Clinical expression

Fifty percent of untreated patients with classic homocystinuria will have a cardiovascular event before the age of 30[4]. Taylor et al.[19] reported that patients with vascular disease with elevated plasma homocysteine levels in the fasting state were more likely to have clinical progression of lower extremity and coronary disease, but not of cerebral vascular disease. In addition, the rate of progression was more rapid in these patients than in patients with normal fasting homocysteine levels. Stampfer et al.[20] reported, in healthy men, an 18 2% increase in relative risk of myocardial infarction for each 1SD increase in fasting homocysteine concentration. Other studies, however, suggest that the homocysteine level after methionine loading, as compared to the fasting level, discriminates more efficiently between patients with or without vascular disease[7,8]. The relative predictive value of fasting versus post-load concentrations can only be determined in a prospective, direct comparison, but such data are not available.

Prevalence

We observed mHH after methionine loading in 25% of patients with peripheral occlusive arterial disease and in 28% of patients with cerebral occlusive disease. In concordance with previous reports we observed a lower prevalence of hyperhomocysteinemia (15%) in the subgroup of patients with a myocardial infarction[6,21]. In the two groups with and without mHH, the distribution of other potential risk factors such as hyperlipoproteinemia, smoking habits, diabetes mellitus and hypertension was not significantly different. There were relatively more women than men among the patients with mHH, however. Possibly the endothelial cells of women are more vulnerable to high homocysteine levels[7]. Although hyperhomocysteinemia is believed to be an independent risk factor[8,22], the interaction of hyperhomocysteinemia with other risk factors needs further investigation.

Therapy

Homocysteine can be either catabolized to cysteine in the transsulfuration pathway by the cystathionine synthase enzyme, with the use of vitamin B_6 as an essential cofactor, or be remethylated to methionine, a reaction that requires folate and vitamin B_{12}, as cosubstrate and cofactor, respectively (Figure 9.1). In addition, homocysteine can be remethylated through the demethylation of betaine (Figure 9.1). In vascular patients with mHH Brattström et al.[12] observed a mean reduction of 39% in plasma total homocysteine concentrations after a methionine load within four weeks of treatment with vitamin B_6 plus folic acid, whereas fasting levels were reduced by 53%. We found similar reductions in post-load (49%; CI 35-57) and fasting (51%; CI 32-64) homocysteine concentrations after 6 to 12 weeks of therapy. In addition, we observed normal post-load and fasting homocysteine levels in 92% and 91% of the patients after 6 weeks of treatment with vitamin B_6 plus folic acid. Six hyperhomocysteinemic patients showed no normalization of the post-load homocysteine levels within 6 weeks. Three patients normalized their post-load homocysteine level after addition with betaine, but 3 other patients also normalized their post-load homocysteine concentrations without reinforcement with betaine. Therefore, the effect of vitamin therapy is probably not maximal after 6 weeks of treatment.

Side effects such as progressive sensory ataxia have been described in patients receiving large doses (up to 6 g) of vitamin B_6 daily[23]. However, no symptoms of neuropathy were found in patients with classic homocystinuria during long-term vitamin B_6 treatment[24]. No serious side effects of treatment with 5 mg folic acid or betaine 6 g daily have yet been reported[25,26].

Limitations

Several limitations of our study should be considered. First, we did not analyze plasma vitamin concentrations (which were within the normal range) as determinants of homocysteine levels either before or after treatment. It is unlikely, however, that such an analysis would affect the main conclusion from this study - that treatment with vitamin B_6 plus folic acid effectively reduces homocysteine concentrations. Second, we did not perform dose-response studies, and the doses of vitamin B_6 and folic acid, although effective and apparently safe, are not necessarily optimal. Third, we did not assess the efficacy of the proposed

treatment in terms of improvement of vascular prognosis. Therefore, long-term intervention studies are needed to investigate whether chronic treatment with vitamin B_6 plus folic acid will reduce the progress of the arteriosclerotic process in vascular patients with mHH.

Lowering of plasma homocysteine in patients with mHH appears to be simple and inexpensive. Our study shows normal responses to methionine loading after 12 weeks of treatment with vitamin B_6 plus folic acid in virtually all patients with mild hyperhomocysteinemia. Therefore, in patients with mHH normalization of homocysteine metabolism is possible within 12 weeks, and, in practice, there seems no need to investigate the effects of therapy before these 12 weeks.

References

1. Mudd SH, Levy HL, Skovby F. Disorders of transsulfuration. In: Scriver CR, Beaudet AL, Sly WS, Valle D (eds). The metabolic basis of inherited disease. McGraw-Hill Inc., New York, 1989;693-734.
2. Boers GHJ. Homocystinuria- a risk factor of premature vascular disease. Clinical Research Series no 3. Dordrecht-Holland/Riverton-USA, Foris Publications, 1986.
3. Wilcken DEL, Wilcken B, Dudman NPB, Tyrell PA. Homocystinuria -the effects of betaine in the treatment of patients not responsive to pyrodoxine. N Eng J Med 1983;309:448-453.
4. Mudd SH, Skovby F, Levy HL, Pettigrew KD, Wilcken B, Pyeritz RE, Andria G, Boers GHJ, Bromberg IL, Cerone R, Fowler B, Gröbe H, Schmidt H, Schweitzer L. The natural history of homocystinuria due to cystathionine ß-synthase deficiency. Am J Hum Genet 1985;37:1-31.
5. Ueland PM, Brattström L, Refsum H. Plasma homocysteine and cardiovascular disease. In: Francis RB (ed). The hemostatic system, endothelial function, and cardiovascular disease. Marcel Dekker Inc., New York, 1992;183-236.
6. Kang SS, Wong PKW, Malinow MR. Hyperhomocyst(e)inemia as a risk factor for occlusive vascular disease. Annu Rev Nutr 1992;2:279-298.
7. Boers GHJ, Smals AGH, Trijbels JMF, Fowler B, Bakkeren JAJM, Schoonderwaldt HC, Kleijer WJ, Kloppenborg PWC. Heterozygosity for homocystinuria in premature peripheral and cerebral occlusive arterial disease. N Engl J Med 1985;313:709-715.
8. Clarke R, Daly L, Robinson K, Naughten E, Cahalane S, Fowler B, Graham I. Hyperhomocysteinemia: an independent risk factor for vascular disease. N Engl J Med 1991;324:1149-1155.
9. Kang SS, Wong PWK, Susmano A, Sora J, Norusis M, Ruggie N. Thermolabile methylenetetrahydrofolate reductase: An inherited risk factor for coronary artery disease. Am J Hum Genet 1991;48:536-545.
10. Dudman NPB, Wilcken DEL, Wang J, Lynch JF, Macey D, Lundberg P. Disordered methionine/homocysteine metabolism in premature vascular disease. Its occurrence, cofactor therapy, and enzymology. Arterioscler Thromb 1993;13:1253-1260.
11. Brattström L, Lindgren A. Hyperhomocyteinemia as a risk factor for stroke. Neur Res 1992;14:81-84.
12. Brattström L, Israelsson B, Norrving B, Bergqvist D, Thörne J, Hultberg B, Hamfelt A. Impaired homocysteine metabolism in early-onset cerebral and peripheral occlusive arterial disease. Effects of pyridoxine and folic acid treatment. Atherosclerosis. 1990;81:51-60.
13. Ubbink JB, Vermaak WJK, Bissbort S. Rapid high-performance liquid chromatographic assay for total homocysteine levels in human serum. J Chromatogr 1991;565:441-446.
14. Dudman NPB, Hicks C, Wang J, Wilcken DEL. Human arterial endothelial cell detachment in vitro: its promotion by homocysteine and cysteine. Atherosclerosis 1991;91:77-83.
15. Harpel PC, Chang VT, Borth W. Homocysteine and other sulfhydryl compounds enhance the binding of lipoprotein(a) to fibrin: A potential biochemical link between thrombosis, atherogenesis, and sulfhydryl compound metabolism. Proc Natl Acad Sci 1992;89:10193-10197.
16. Lentz SR, Sadler JE. Inhibition of thrombomodulin surface expression and protein C activation by the thrombogenic agent homocysteine. J Clin Invest 1991;88:1906-1914.
17. Starkebaum G, Harlan JM. Endothelial cell injury due to copper-catalyzed hydrogen peroxide generation from homocysteine. J Clin Invest 1986;77:1370-1376.
18. Stamler JS, Osborne JA, Jaraki A. Adverse vascular effects of

homocysteine are modulated by endothelium-derived relaxing factor and related oxides of nitrogen. J Clin Invest 1993;91:308-318.
19. Taylor LM, DeFrang RD, Harris EJ, Porter JM. The association of elevated plasma homocyst(e)ine with progression of symptomatic peripheral arterial disease. J Vasc Surg 1991;13:128-136.
20. Stampfer MJ, Malinow MR, Willett WC, Newcomer LM, Upson B, Ullman D. A prospective study of plasma homocysteine and risk of myocardial infarction in us physicians. JAMA 1992;268:877-881.
21. Kang S-S, Wong PWK, Cook HY, Norusis M, Messner JV. Protein-bound homocyst(e)ine. A possible risk factor for coronary artery disease. J Clin Invest 1986;77:1482-1486.
22. Mölgaard J, Malinow MR, Lassvik C, Holm AC, Upson B, Olsson AG. Hyperhomocyst(e)inaemia: an independent risk factor for intermittent claudication. J Intern Med 1992;231:272-279.
23. Schaumberg H, Kaplan J, Windebanke A, Vick N, Rasmus S, Pleasure D, Brown MJ. Sensory neuropathy from pyridoxine abuse. N Eng J Med 1983;309:445-448.
24. Mpofu C, Alani SM, Whitehouse C, Fowler B, Wraith JE. No sensory neuropathy during pyridoxine treatment in homocystinuria. Arch Dis Child 1991;66:1081-1082.
25. Brody T, Shane B, Stokstad ELR. Folic acid. In: Machlin LJ (ed). Handbook of vitamins, nutritional, biochemical, and clinical aspects. Marcel Dekker Inc., New York and Basel, 1984;459-496.
26. Friedrich W. Folic acid and unconjugated pteridines. In: Friedrich W, (ed). Vitamins. Walter de Gruyter Inc., New York, 1988;619-752.

THIAMINE (VITAMIN B_1) SUPPLEMENTATION DOES NOT REDUCES FASTING BLOOD HOMOCYSTEINE CONCENTRATION IN MOST HOMOZYGOTES FOR HOMOCYSTINURIA

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Abstract

Homozygotes for homocystinuria due to cystathionine synthase (CS) deficiency accumulate homocysteine and methionine in their blood and tissues. High-dose pyridoxin, folic acid, vitamin B_{12}, or betaine are therapeutical options to lower the elevated homocysteine concentration. These compounds stimulate the transsulphuration or remethylation of homocysteine. Despite such treatment, elevated blood homocysteine concentrations may persist in many homocystinurics. Therefore, it is warranted to study alternative regimen to reduce the blood homocysteine concentration in homocystinurics. Apart from entering the transsulphuration pathway, methionine can be catabolized via the transamination pathway, by conversion into 4-methylthio-2-oxobutyrate (MTOB), followed by oxydative decarboxylation of MTOB to 3-methylthiopropionate. Thiamine pyrophosphate, the active form of thiamine, is a cofactor of the supposed rate-limiting oxidative decarboxylation in the transamination of methionine. The effect of thiamine administered in 2 or 3 daily doses of 25 mg orally, was studied in nine homozygote CS deficient patients. Methionine levels decreased in 6 out of 9 patients In 8 out of 9 patients, however, the levels of plasma homocysteine remained virtually unchanged, as did the serum transamination metabolites in all patients. We conclude that vitamin B_1 cannot be used as an additional homocysteine-lowering treatment in most homozygotes for homocystinuria.

Introduction

Patients with classic homocystinuria due to cystathionine synthase (CS) deficiency accumulate homocysteine and methionine in their blood and tissues. The disease is clinically characterized by arteriosclerosis, thromboembolism, eye-lens luxation, marfanoid features, osteoporosis, and mental retardation. Therapy is based on reduction of the hyperhomocysteinemia by stimulation of residual CS activity by high levels of the cofactor pyridoxal-5'-phosphate[1]. About 60% of these patients respond to pyridoxine. In poor or non-pyridoxine responsive patients, treatment can be extended with folic acid, vitamin B_{12} or betaine supplementation, which enhances the homocysteine remethylation (Figure 10.1)[1]. As an ultimative option, dietary methionine restriction may be mandatory to reduce the blood homocysteine concentration. Despite this variety of therapeutical options, severely elevated homocysteine concentrations may persist in homozygotes for homocystinuria[2,3]. It is therefore warranted to study additional possibilities to reduce the blood homocysteine concentration in homocystinurics.

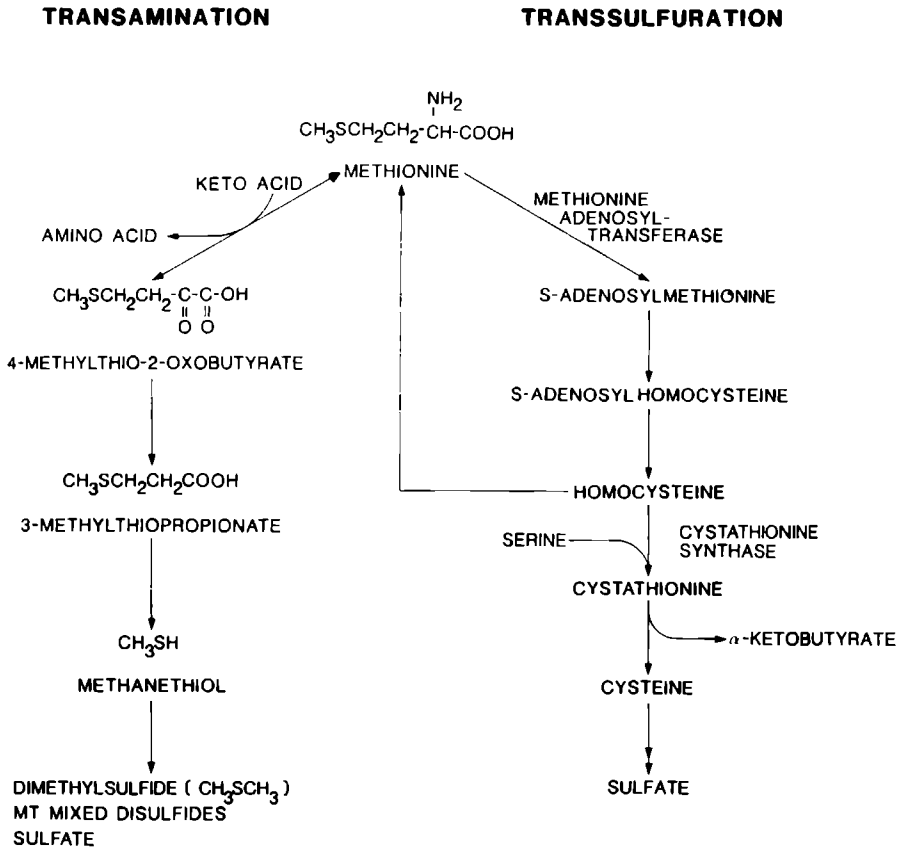

Figure 10.1. Metabolism of methionine.

Apart from entering the transsulphuration pathway, methionine can also be degraded via the transamination pathway by conversion into 4-methylthio-2-oxobutyrate (MTOB), followed by oxydative decarboxylation of MTOB to 3-methylthiopropionate (Figure 10.1)[4]. In the latter reaction thiamine pyrophosphate, the active form of thiamine, is a cofactor.

We previously demonstrated in an in vitro study that thiamine pyrophosphate stimulates the degradation of methionine via the transamination pathway in rat liver homogenates[5]. The same result was obtained in human liver homogenates (Blom; unpublished results). In this study, the methionine and homocysteine-lowering effect of thiamine (vitamin B_1) was tested in nine homozygote CS deficient patients.

Materials and methods

Homozygotes for homocystinuria due to CS deficiency

The criteria for selection of homozygotes for homocystinuria to enter this study were: regulary visiting our hospital, known with good compliance to their homocysteine-lowering treatment, prolonged elevated fasting homocysteine levels in blood despite homocysteine-lowering treatment for at least 2 years, and at least 14 years of age. Homozygosity for homocystinuria in nine patients was proven by hypermethioninemia, severe hyperhomocysteinemia as well as a near to absent CS activity in cultured skin fibroblasts[3]. None of them were on a regimen of methionine restriction, except one (patient number 9; Table 10.1). In addition to their conventional homocysteine-lowering treatment, 6 patients received 25 mg of thiamine hydrochloride for 6 weeks three times daily and 3 patients twice daily (Table10.1: patient number 2, 6 and 7). The latter 3 patients received only in total 50 mg thiamine hydrochloride conforming with their conventional homocysteine-lowering treatment which was also twice daily. Patients 3 and 4 are brothers, as well as patients 6 and 7 (Table 10.1).

Methionine and homocysteine assay techniques

Fasting EDTA venous blood samples were centrifuged immediately after puncture and stored at -20 °C until analysis. Methionine concentrations were determined on a LC 2000 amino acid analyzer (Biotronik Wissenschaftliche Geräte, Munich, Germany)[6]. The total homocysteine concentrations (free plus protein bound) were measured by a technique based on high-performance liquid chromatography (HPLC) and fluorescent detection[7].

Transamination metabolites assay techniques

The degradation of methionine via the transamination pathway before and after treatment was studied by quantification in serum by the sum of the transamination metabolites 4-methylthio-2-oxobutyrate and the mixed disulphides of methanethiol ($R\text{-}S\text{-}S\text{-}CH_3$). The blood concentrations of these metabolites are low and near the detection limit, and because of reasons of accuracy, we prefer to measure the total sum of these transamination metabolites. This technique is based on gas chromatography (Packard type 429; Packard-Becker, Delft, the Netherlands), supplied with a sulphur-specific flame-photometric detector (Packard, model 906)[8,9]. Details of the transamination metabolites measurement have been reported previously[10].

Vitamin B_1 measurements

Total thiamine concentrations were determined by a modified procedure of the reversed-phase ion-pair HPLC technique described by Wielders and Mink[11]. Whole blood specimens are deproteinized with perchloric acid, followed by acid phosphatase hydrolysis of thiamine tri-, di- and monophosphate to thiamine, and post-column derivatisation of total thiamine with $K_3Fe(CN)_6$ to thiochrome which is quantified fluorimetrically at excitation and emission wave lenghts of 364 nm and 462 nm, respectively. Due to preparation steps prior to HPLC, total analysis time of a blood specimen takes approximately 20 hours while the HPLC run itself, requires less than 10 minutes. The lower limit of detection of the technique is 2.3 nmol thiamine per liter and excellent linear standard dose-responses is obtained up to 400 nmol/l. At mean concentrations of 123 nmol/L, the precision of the technique revealed intra-assay and inter-assay coefficients of variation of 4.8% (9 assay runs) and 6.8% (100 consecutive assay runs), respectively.

Statistical analysis

Results are given as mean ± SD. The two tailed Wilcoxon rank sum test was used in assessing statistical significance.

Results

The mean age ± SD of all 9 homozygotes for homocystinuria was 24.4 ± 6.2 year (range 14 to 34 year); the mean ± SD duration of homocysteine-lowering treatment was 9.0 ± 2.4 year (range 5 to 17 year). The mean ± SD body weight was 72.3 ± 10.6 kg (range 52.5 to 88.0 kg) and the mean ± SD mg thiamine dosage per kg body weight for the patients was 0.90 ± 0.25 mg thiamine supplementation per kg body weight (range 0.48 to 1.43).

The vitamin B_1 plasma concentration in the homozygotes for homocystinuria increased from 123 ± 30 (mean ± SD) nmoles/L (n = 7) before thiamine treatment to 207 ± 38 nmoles/L (n = 7) after 6 weeks of thiamine treatment (Table 10.1). The fasting blood methionine concentration decreased from 194 ± 127 μmoles/L (mean ± SD) before thiamine treatment to 128 ± 94 μmoles/L after thiamine treatment (n = 9) ($p < 0.06$). The mean fasting plasma homocysteine concentrations did not differ significantly before and after thiamine therapy, i.e. 91 ± 25 μmmoles/L versus 83 ± 22 μmoles/L. Only one out of the nine thiamine treated patients showed a considerable lowering of the homocysteine concentration simultaneously with a major decrease of the methionine level (Table 10.1: patient number 1). Currently, this patient continues the vitamin B_1 supplementation for 3 years, and the basal total homocysteine concentration at the most recent determination is now as low as 34 μmoles/L. The level of the transamination metabolites after thiamine did not increase in the patients. In fact, these levels remained low or undetectable in 6 patients and even decreased slightly in 3 other patients (Table 10.1). Even in the single patient with a decrease of the homocysteine concentration since the start with thiamine supplementation, the level of the transamination metabolites remained unchanged.

Table 10.1. The fasting blood concentrations of total homocysteine, methionine, thiamine (vitamin B_1) and transamination metabolites in 9 cystathionine synthase deficient patients before ($-B_1$) and after ($+B_1$) 6 weeks of thiamine treatment. Vitamin B_6 (B_6) was given in a daily dose in mg as indicated; folic acid (FA) was given in a daily dose of 5 mg; vitamin B_{12} (B_{12}) was given in a two monthly dose of 1 mg intramuscularly; betaine was given in a daily dose of 6 g; methionine poor regimen (MPR); np = not performed; bd = below level of detection

Patient	Homocysteine µmol/L		Methionine µmol/L		Thiamine nmol/L		Transamination µmol/L		Therapy
	$-B_1$	$+B_1$	$-B_1$	$+B_1$	$-B_1$	$+B_1$	$-B_1$	$+B_1$	
1.	143	48	98	30	98	170	0.3	0.3	750B_6, B_{12}
2.	46	49	43	42	96	130	0.3	0.3	400B_6, FA, B_{12}
3.	81	82	291	123	110	190	0.6	0.3	750B_6, FA, betaine
4.	82	79	94	54	120	210	bd	bd	750B_6, FA, betaine
5.	58	56	161	58	100	210	0.3	0.3	750B_6, FA, betaine
6.	113	101	222	93	np	np	0.4	0.2	500B_6, FA, betaine
7.	82	120	139	188	np	210	bd	bd	500B_6, FA, betaine
8.	85	103	57	72	110	np	0.3	0.3	750B_6, FA, B_{12}, betaine
9.	128	109	643	490	230	330	1.0	0.7	250B_6, FA, betaine, MPR
mean ±SD	91 ±25	83 ±22	194 ±127	128 ±94	123 ±30	207 ±38			
normal	5 to 18		16 to 47		47 to 142		bd to 1.2		

Discussion

Methionine degradation via the transamination pathway occurs in human but is probably of minor quantitative importance[10]. However, patients with hypermethioninemia due to methionine adenosyltransferase (MAT) deficiency do degrade quantitative amounts of methionine via the transamination pathway[12,16], whereas patients with hypermethioninemia due to cystathionine synthase (CS) deficiency do not, despite elevated methionine levels[6]. The functional impairment of methionine transamination may be due to the different biochemical level of the blockade in the methionine metabolism or due to the treatment of CS deficient patients by pharmacological amounts of vitamins, in particularly pyridoxine[6]. The present study was performed because of the theoretical possibility to lower homocysteine concentrations by enhancement of methionine degradation via the transamination pathway in CS deficient patients. Previous in vitro studies with rats[5] and human homogenates [Blom H, unpublished data] have shown that thiamine pyrophosphate stimulated the methionine degradation via the transamination pathway 2.5 fold. This active form of thiamine is a cofactor of the branched-chain 2-oxo-acid dehydrogenase complex which catalyses the oxydative decarboxylation of 4-methylthio-2-oxobutyrate into 3-methylthiopropionate[13,14]. This decarboxylation is supposed to be the rate-limiting reaction in the transamination of methionine[4].

Thiamine was administered in 9 homozygotes for homocystinuria in addition to their conventional homocysteine-lowering treatment. All these patients except one, demonstrated virtually unchanged levels of plasma homocysteine and serum transamination metabolites, despite the observed reduction of their methionine concentrations. Very recently, it has been reported that transamination in hypermethioninemic children is abnormally elevated only when plasma methionine levels exceeded 300 or 350 μM[15,16]. In the present study, only patient number 9 (Table 10.1) has a methionine level above this methionine level, and indeed, his transamination metabolites were higher than in the other 8 investigated homozygotes for homocystinuria, but still much lower than those of MAT deficient patients with comparable methionine levels[6,16]. But even his serum transamination metabolites concentration did not increase after the supplementation of thiamine.

We have no straightforward explanation for the reduction of the methionine concentration in most of the homocystinuric patients due to thiamine supplementation. The flux of methionine degradation through the transamination pathway can be decreased by glutamine, glutamic acid, alanine or leucine in the presence of 4-methylthio-2-oxobutyrate[17]. Thiamine is involved as a cofactor in the oxydative decarboxylation in the degradation of many amino acids, including the four mentioned above. Theoretically, thiamine administration may decrease the levels of glutamine, glutamic acid, alanine and leucine, and indirectly stimulate methionine degradation via its transamination pathway. However, the serum transamination metabolites of methionine were not elevated during thiamine administration. But, the levels of these metabolites may have remained unchanged despite an increased methionine degradation through the transamination pathway. Studies using stable isotopes of methionine could clarify this matter.

In conclusion, orally administered thiamine lowered the homocysteine level in only one out of the nine homocystinuric patients studied despite the reduction of the methionine concentration in most patients. Therefore, vitamin B_1 appears to

be only a minor alternative option for homocysteine-lowering treatment next to vitamin B_6, B_{12}, folic acid, and betaine in homozygotes for homocystinuria.

Acknowledgements

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References

1 Mudd SH, Levy HL, Skovby F. Disorders of transsulfuration. In: Scriver CR, Beaudet AL, Sly WS, Valle D (eds). The metabolic basis of inherited disease. McGraw-Hill Inc., New York, 1989,693-734.

2 Boers GHJ, Smals AGH, Drayer JIM, Trijbels JMF, Leermakers AI, Kloppenborg PWC. Pyridoxine treatment does not prevent homocystinemia after methionine loading in adult homocystinuria patients. Metabolism 1983;32:390-397.

3 Boers GHJ. Homocystinuria, a risk factor of premature vascular disease. Clinical Research Series no 3. Dordrecht-Holland/Riverton-USA, Foris Publications, 1986.

4 Jones MA, Yeaman SJ Oxidative decarboxylation of 4-methylthio-2-oxobutyrate by branched-chain 2-oxoacid dehydrogenase complex. Biochem J 1986;237:621-623.

5. Blom HJ, Boers GHJ, Tangerman A, Gahl WA, Trijbels JMF. Alternative methionine degradation via the transamination pathway. an option for therapy for homocystinuria due to cystathionine synthase deficiency J Inher Metab Dis 1991,14 375-378.

6. Blom HJ, Boers GHJ, Trijbels JMF, van Roessel JJM, Tangerman A. Cystathionine synthase deficient patients do not utilize the transamination pathway of methionine to reduce hypermethioninemia and homocysteinemia Metabolism 1989;38.577-582.

7. te Poele-Pothoff MTWB, van den Berg M, Franken DG, Boers GHJ, Jakobs C, De Kroon IFI, Eskes TKAB, Trijbels JMF, Blom HJ. Three different methods for the determination of total homocysteine in plasma. Ann Clin Biochem 1995;32.218-220.

8. Tangerman A, Meuwese-Arends MT, van Tongeren JHM. New methods for the release of volatile sulphur compounds from human serum: in determination by Tenax trapping and gas chromatography and its application in liver disease. J of Lab and Clin Med 1985;106:175-182.

9. Tangerman A, Meuwese-Arends MT, van Tongeren JHM. A new sensitive assay for measurements volatile sulfur compounds in human breath by Tenax trapping and gas chromatography. Clin Chim Acta 1983;130·103-110.

10. Blom HJ, Boers GHJ, van den Elzen JPAM, Gahl WA, Tangerman A Transamination of methionine in humans Clin Sci 1989;76:43-49

11 Wielders JPM, Mink CJK. Quantitative analysis of total thiamine in human blood, milk and cerebrospinal fluid by reversed-phase ion-pair high-performance liquid chromatography. J Chromatography 1983,277·145-156.

12. Gahl WA, Bernardini I, Finkelstein JD, Tangerman A, Martin JJ, Blom HJ, Mullen KK, Mudd SH. Transsulfuration in an adult with hepatic methionine adenosyltransferase deficiency. J Clin Invest 1988;81·390-397.

13. Yeaman SJ The mammalian 2-oxoacid dehydrogenases· a complex family. Trends Biochem Sci 1986;11:293-296.

14 Livesey G, Lund P. Methionine metabolism via the transamination pathway in the rat liver. Biochem Soc Trans 1980;8:540-541.

15. Tangerman A, Mudd SH, Levy HL. Transamination of methionine in children with hypermethioninemia. In: Mato JM, Caballero A (eds). Methionine metabolism: molecular mechanisms and clinical implications. Bouncopy Inc., Madrid, 1994;211-216.

16 Mudd SH, Levy HL, Tangerman A, Boujet C, Buist N, Davidson-Mundt A, Hudgins L, Oyanagi K, Nagao M, Wilson WG Isolated persistent hypermethioninemia. Am J Hum Gen 1995,57:882-892

17 Scislowski PWD, Pickard K. The regulation of transaminative flux of methionine in rat liver mitochondria. Arch of Biochem and Biophys 1994,314 412-416.

PART 3
CLINICAL EFFECT OF TREATMENT OF HYPERHOMOCYSTEINEMIA

HYPERHOMOCYSTEINAEMIA AND ENDOTHELIAL DYSFUNCTION IN YOUNG PATIENTS WITH PERIPHERAL ARTERIAL OCCLUSIVE DISEASE

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Abstract

Hyperhomocysteinaemia, defined as an abnormally high plasma homocysteine concentration after an oral methionine load, is common in young (≤ 50 yr) patients with peripheral arterial occlusive disease. It is thought to predispose to atherosclerosis by injuring the vascular endothelium. Treatment with pyridoxine and/or folic acid may lower plasma homocysteine levels. In mildly hyperhomocysteinaemic patients with peripheral arterial occlusive disease, we studied the effect of daily treatment with pyridoxine (250 mg) plus folic acid (5 mg) on homocysteine metabolism (i.e., plasma concentrations in the fasting state and after methionine loading; in 48 patients) and on endothelial function (in 18 patients). Endothelial function was estimated as the plasma concentrations of the endothelium-derived proteins, von Willebrand factor (vWF), thrombomodulin (TM), and tissue-type plasminogen activator (tPA). At baseline, fasting homocysteine levels were above normal in 24 of the 48 patients (50%); post-load levels, by definition, in 100%. After 12 weeks of treatment, fasting and post-load levels were normal in 98 and 100%, respectively.

Endothelial function was assessed in 18 patients who completed one year of treatment At baseline, median vWF (235%) and TM (57.1 ng/ml) levels were above normal At follow-up, vWF levels had decreased to 170% (p0.01) and TM levels to 49 ng/ml (p = 0.04) tPA levels were normal at baseline and did not change.

Endothelial dysfunction is present in young patients with peripheral arterial occlusive disease and hyperhomocysteinaemia. Pyridoxine plus folic acid treatment normalises homocysteine metabolism in virtually all patients, and appears to ameliorate endothelial dysfunction.

Introduction

Mild hyperhomocysteinaemia (mHH) is an independent risk factor for peripheral arterial occlusive disease[1-4]. Studies in animals and *in vitro* indicate that high plasma concentrations of homocysteine, derived from demethylation of dietary methionine, may predispose to atherosclerosis by injuring the vascular endothelium, which results in endothelial dysfunction[5-9]. Pyridoxine and/or folic acid supplementation have been shown to reduce plasma homocysteine concentrations in mildly hyperhomocysteinaemic patients with peripheral occlusive disease[10], but it is not known to what extent such treatment normalises homocysteine metabolism as estimated by plasma homocysteine concentrations in the fasting state and after an oral methionine load. It is also unknown whether endothelial dysfunction is present in these patients, and, if so, whether treatment aimed at normalising homocysteine metabolism improves endothelial function.

We assessed endothelial function in young patients with peripheral arterial occlusive disease and hyperhomocysteinaemia as defined by an abnormal plasma concentration after methionine loading[1]. A gold standard for endothelial dysfunction is not available; we, therefore, measured plasma levels of 3 endothelium-derived proteins involved in the regulation of haemostasis, i.e., von Willebrand factor (vWF), thrombomodulin (TM) and tissue-type plasminogen activator (tPA), because *in vitro* and *in vivo* data suggest that endothelial injury is associated with increased plasma levels of these proteins[11-18]. In addition, we investigated the effects of treatment with pyridoxine plus folic acid on homocysteine metabolism and endothelial function.

Patients and methods

Patients

From November, 1991, to April, 1993, 205 consecutive patients presenting with peripheral arterial occlusive disease at age 50 or younger were recruited from the Department of Vascular Surgery at the Free University Hospital, Amsterdam. Symptomatic peripheral arterial occlusive disease was defined as intermittent claudication and/or ischaemic ulceration, rest pain and gangrene, and/or amputation for ischaemia, and was confirmed by Doppler and/or angiographic studies. After obtaining informed consent, clinical and laboratory data were collected.

Blood pressure was measured after 15 min of supine rest without altering antihypertensive regimens. Diabetes was defined according to WHO criteria, and known diabetes duration was recorded. Patients were classified as non-smokers (those who did not smoke cigarettes, cigars, or pipe) or smokers (all others). Drug use was also recorded. After an overnight fast, blood was drawn for measurement of serum total cholesterol (measured enzymatically), creatinine (modified Jaffé reaction), glucose (glucose oxidase method), and endothelial function parameters (specified below), and an oral methionine loading test was performed to detect hyperhomocysteinaemia. The clinical data were collected and laboratory assays performed by personnel "blinded" to the presence of treatment with pyridoxine plus folic acid.

Methionine loading test

Plasma levels of homocysteine were determined in the fasting state and 6 hours after an oral methionine load (0.1 g/kg). Plasma homocysteine levels were

measured as total homocysteine by using HPLC with fluorescence detection[19]. Normal fasting and post-load values measured in our laboratory are <18 and <51 μmol/L in men (n=23), <15 and <49 μmol/L in premenopausal women (n=56), and <19 and <69 μmol/L in postmenopausal women (n=20). Deficiencies of vitamin B_{12}, pyridoxine and folic acid were excluded by measuring serum levels. Normal values are >120 pmol/L for vitamin B_{12}, >19 nmol/L for pyridoxine, and >3.4 nmol/L for folic acid.

Two hundred and five patients were so tested; 48 (23%) had hyperhomocysteinaemia as defined by an abnormal post-load plasma concentration[1]. Table 11.1 shows the clinical features of these patients. All had normal renal and hepatic function.

Main outcome measures

The 48 hyperhomocysteinaemic patients were all treated with pyridoxine (250 mg daily) plus folic acid (5 mg daily). The effect on fasting and post-load homocysteine levels was studied after 6 weeks, and, in patients in whom the post-load plasma concentration at 6 weeks was not in the normal range, again 6 weeks later. Parameters of endothelial function, and the effect of treatment on these parameters, were investigated in all patients who, in June, 1993, had completed at least one year of treatment (n=18), a period chosen to allow a reasonable amount of time for the effect of treatment on endothelial function to become manifest.

Endothelial function: The plasma concentrations of von Willebrand factor antigen (vWF)[20], thrombomodulin (TM; Asseraduron Trombomoduline, Diagnostica Stago, France)[18], and tissue-type plasminogen activator antigen (tPA; Imulyse tPA, Biopool, Sweden)[21], were measured by enzyme-linked immunosorbent assays. The plasma vWF level is expressed as a percentage of normal pooled plasma, the antigen level of which is defined as 100% (normal range, 50-150%[13]). For TM and tPA, normal ranges are 16.5-47.5 ng/ml and 1.84-9.80 ng/ml, respectively, as obtained in a control group (n=21) matched for age with the 18 hyperhomocysteinaemic patients in whom TM and tPA were measured. All blood samples were taken between 8 and 9 a.m., after an overnight fast. We were careful to avoid acute increases in the concentrations of these proteins associated with exercise, smoking, prolonged venous occlusion, hypoglycaemia, and acute illness. The intra- and interassay variation of the vWF, TM and tPA assays is less than 10%; the within person day-to-day variability of vWF is about 10%; that of tPA, about 15% (unpublished).

Statistical analysis

Data are given as mean (SD) or as median (range), unless indicated otherwise. Parametric and non-parametric tests were used as appropriate. Paired tests were used for comparing baseline with post-treatment data. We used univariate analysis to study the relation between endothelial function and possible determinants thereof: fasting and post-load homocysteine levels as well as 'classic' risk factors for atherosclerotic vascular disease (age, sex, smoking habits, systolic and diastolic blood pressure, and serum cholesterol; diabetes was not included in the analysis because there was only one diabetic among the 18 patients in the endothelial function study). The relationship of these 'classic' risk factors with endothelial

function parameters was assessed both at baseline and at follow-up (ie, at one year). In addition, we studied the relationship between homocysteine levels (fasting and post-load) and endothelial function parameters at baseline, and between homocysteine levels after treatment (ie, at 6 weeks) and endothelial function parameters at follow-up (ie, at one year). All testing was two-tailed with 0.05 as the level of significance.

Results

The patients reported no adverse effects of the pyridoxine plus folic acid treatment. No new vascular events occurred during follow-up, either in the 48 patients followed for 6 to 12 weeks or in the subgroup of 18 patients followed for ≥ one year. Mean (SD) fasting and post-load plasma homocysteine levels were 20.7 (14.7) and 73.8 (22.3) μmol/L before treatment, and 9.6 (3.6) and 36.8 (9.1) μmol/L after treatment ($p < 0.001$ for both comparisons; Figure 11.1). By definition, post-load homocysteine concentrations before treatment were abnormal in all patients; in contrast, fasting concentrations were within the normal range in 24 of the 48 patients (50%). After 6 weeks of treatment, the fasting and post-load homocysteine concentrations were within the normal range in 47 (98%) and 45 (94%) of 48 patients, respectively. After 12 weeks, these figures were 47 (98%) and 48 (100%).

Table 11.2 shows the clinical characteristics of the 18 patients who completed at least one year of treatment (in whom endothelial function was assessed). At baseline, vWF and TM levels were above normal, but tPA levels were in the normal range. At follow-up, plasma vWF concentrations had decreased from median 235 to 170% ($p = 0.01$) and plasma TM concentrations from 57.1 to 49.0 ng/ml ($p = 0.04$). Plasma tPA levels had not changed (6.9 ng/ml at baseline versus 6.5 ng/ml at follow-up [$p = 0.91$]; Table 11.2, Figure 11.2).

With regard to the classic risk factors, significant relationships were observed at baseline (but not at follow-up) between age and tPA ($r = 0.57$, $p = 0.01$), serum cholesterol and vWF ($r = 0.48$, $p = 0.04$), and smoking habits and TM (smokers had lower TM levels than non-smokers (49 [29-72] versus 70 [48-113] ng/ml; $p = 0.03$). The *fasting* homocysteine plasma levels at baseline and after 6 weeks of treatment were not related to endothelial function parameters at baseline and follow-up, respectively, except for the homocysteine level at 6 weeks and the tPA level at follow-up ($r = 0.44$, $p = 0.06$). In contrast, the *post-load* homocysteine plasma levels at baseline and after 6 weeks of treatment showed trends towards significant relationships with the endothelial function parameters at baseline and follow-up, respectively: vWF (baseline: $r = 0.36$, $p = 0.14$; follow-up: $r = 0.69$, $p = 0.001$, Figure 11.3), TM (baseline: $r = 0.73$, $p = 0.001$; follow-up: $r = 0.35$, $p = 0.15$), and tPA (baseline: $r = 0.06$, $p = 0.8$; follow-up: $r = 0.38$, $p = 0.12$).

Table 11.1. Clinical characteristics of patients with peripheral arterial occlusive disease and hyperhomocysteinaemia. Data are mean (SD) unless otherwise indicated.

N (M/F)	48 (17/31)
Age (yr)	43.9 (1.1)
Blood pressure (mmHg)	138/87 (20/20)
Smokers	32 (68%)
Serum cholesterol (mmol/L)	6.3 (1.2)
Diabetes mellitus	2 (4%)
Serum creatinine (μmol/L)	85 (21)

Table 11.2. Characteristics of the hyperhomocysteinaemic patients who completed one year of treatment. Data are mean (SD) unless otherwise indicated. § p = 0.01, ¶ p = 0.04.

	Baseline	Follow-up
N (M/F)	18 (6/12)	
Age (yr)	43.6 (1.2)	44.6 (1.2)
Follow-up duration (mo)	12.1 (12-13)	
Blood pressure (mmHg)	142/91 (18/26)	141/86 (18/10)
Serum cholesterol (mmol/L)	6.3 (0.2)	6.0 (0.4)
Smoking (Y/N)	13/5	11/7
Diabetes mellitus	1 (5.6%)	1 (5.6%)
von Willebrand factor (%)	235	170 §
Thrombomodulin (ng/ml)	57.1	49 ¶
Tissue-type plasminogen activator(ng/ml)	6.9	6.5

Discussion

In keeping with earlier reports[1-4], our results indicate a high prevalence of mHH among young patients with peripheral arterial occlusive disease. Such patients show evidence of endothelial dysfunction as estimated by vWF and TM plasma concentrations. Endothelial dysfunction is thought to play an important role in atherogenesis. Treatment with pyridoxine plus folic acid normalises homocysteine metabolism in virtually all patients, both in terms of the fasting homocysteine plasma level and the level after an oral methionine load.

Treatment with pyridoxine and folic acid is based on their involvement in homocysteine metabolism. Furthermore, either agent has been shown to lower the grossly elevated homocysteine plasma levels observed in classic homocystinuria, an inborn error of metabolism characterised by premature atherosclerosis and venous and arterial thromboembolism[4]. Classic homocystinuria is usually caused by homozygous deficiency of cystathionine synthase, an enzyme involved in theconversion of homocysteine to cystathionine. The active form of pyridoxine, pyridoxal phosphate, is a cofactor in this reaction. Its homocysteine-lowering effect is thought to be due to stimulation of cystathionine synthase activity. In contrast, folic acid reduces the plasma homocysteine concentration by enhancing its remethylation to methionine[4]. Therefore, the effects of these treatment modalities can theoretically be expected to be additive.

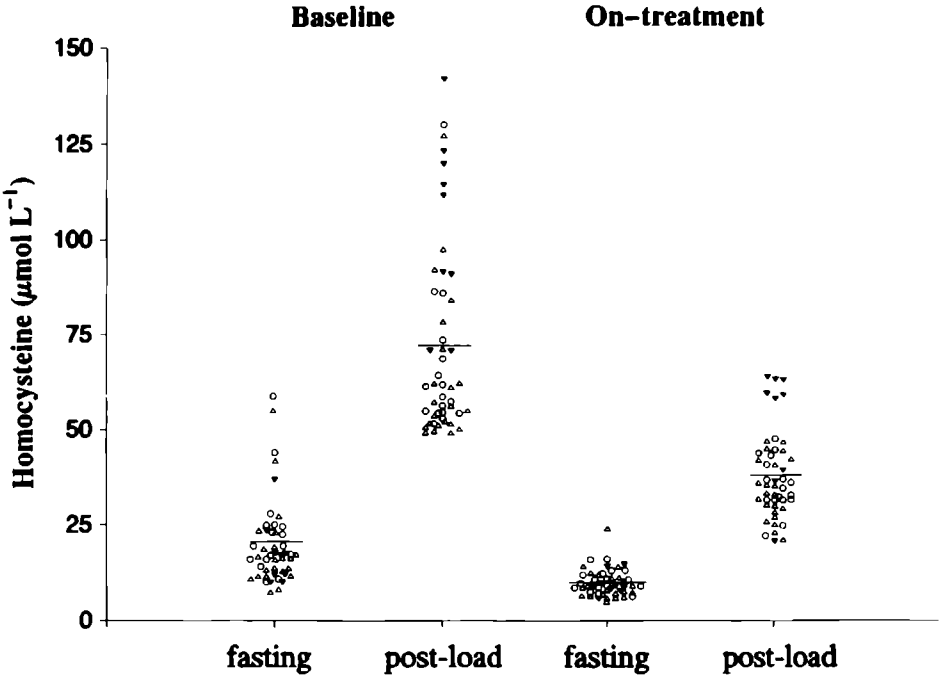

Figure 11.1. Plasma homocysteine concentrations (fasting and after methionine loading) before and on treatment (6 weeks in 45 patients and 12 weeks in 3 patients) with pyridoxine plus folic acid. o, men; ▵, premenopausal women; ▾, postmenopausal women.

Our patients were selected for having mHH, which may be due to heterozygous cystathionine synthase deficiency[1], or to other metabolic defects[4,22]. Reliable and convenient methods to directly measure such deficiencies are not available at present. Instead, methionine loading, which stresses the pathways involved in homocysteine metabolism, can be used as a diagnostic test to uncover abnormalities of homocysteine handling. It is not clear which estimate of homocysteine metabolism, fasting[3,23] or post-load[1,2] level, should be chosen to guide treatment, because it is unknown which is the better predictor of atherosclerotic disease. We, therefore, measured both and observed no major differences in the metabolic efficacy of pyridoxine plus folic acid treatment whether expressed as fasting or as post-load homocysteine concentrations.

Importantly, we found such treatment to be associated with improvement, although not normalisation, of endothelial function, suggesting that hyperhomocysteinaemia-associated endothelial dysfunction might be reversible. Homocysteine, a highly reactive sulphur-containing amino acid, is thought to damage endothelial cells by several mechanisms, eg, generation of hydrogen

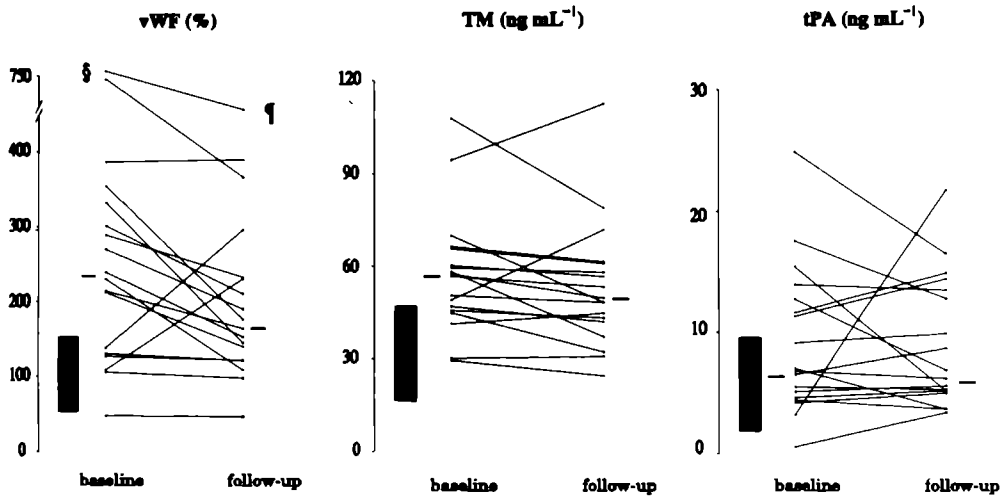

Figure 11.2. Endothelial function parameters before and after one year of treatment with pyridoxine plus folic acid. vWF = von Willebrand factor; TM = thrombomodulin; tPA = tissue-type plasminogen activator. §,¶ = values not to scale, of + 716 and + 727 (§), and + 454 (¶). The black rectangles indicate the normal ranges.

peroxide[7] and depletion of nitric oxide-mediated detoxification of homocysteine[9]. In addition, as hyperhomocysteinaemia is often due to genetic defects in the enzymes that regulate homocysteine metabolism[1,2,4,22], and because these defects are also present in endothelial cells[8], the endothelium of these persons may be especially vulnerable to homocysteine toxicity[6]. Endothelial dysfunction is a central feature of current models of atherogenesis[24]. Increased vWF and TM plasma concentrations probably reflect ongoing endothelial injury[11-18]. Furthermore, high plasma vWF levels have been shown to predict a poor cardiovascular prognosis in survivors of myocardial infarction[12] and in patients with non-insulin-dependent diabetes mellitus[13] The nature of the link between atherosclerosis and vWF and TM is not known with certainty. High vWF and TM probably are markers of the presence of endothelial injury and the process of atherogenesis. In addition, high plasma vWF levels may have functional significance because vWF enhances platelet adhesion and coagulation, the latter by stabilising factor VIII. TM, a membrane-bound protein, contributes to the inhibition of thrombin generation, thereby establishing an important local control of the coagulation cascade. Elevated TM plasma levels may reflect decreased binding to the cell membrane[15], thus

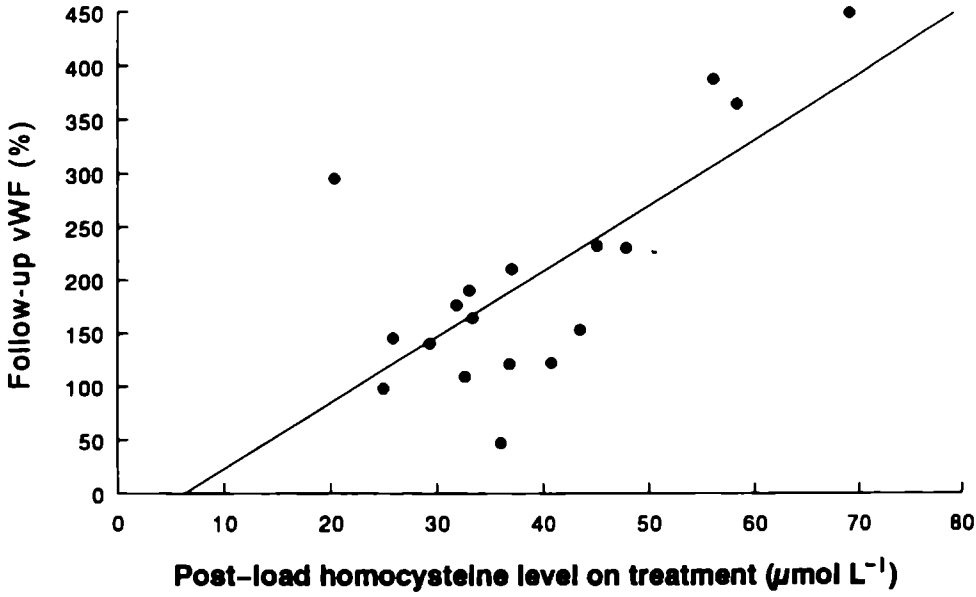

Figure 11.3. von Willebrand factor after one year of treatment with pyridoxine plus folic acid as a function of the post-methionine load plasma homocysteine concentration. r = o.69; p < 0.001.

allowing enhanced thrombin activity, or, alternatively, may result from homocysteine-induced increased synthesis and turnover[25]. The precise mechanism by which hyperhomocysteinaemia increases vWF and TM plasma concentration is unknown. *In vitro* studies have suggested that homocysteine might *decrease* vWF secretion[26] and TM expression[27], findings that indicate major differences between the *in vitro* and *in vivo* situation.

The normal plasma level of tPA, an important regulator of fibrinolysis, suggests that endothelial function was not altered in this respect. Interpretation is limited, however, by the fact that we studied a relatively small group of patients. In addition, we did not measure tPA's inhibitor, PAI-1, which together with tPA is thought to determine fibrinolytic capacity.

Other limitations of our study should also be considered. First, the treatment was neither randomised nor controlled. It appears unlikely, however, that the substantial effect of treatment on homocysteine levels was a chance finding, as such levels are known to be quite stable over time, whereas other conditions known to affect homocysteine metabolism were excluded[4]. In addition, although a placebo-controlled trial would obviously be the most preferable study design, our experience with symptomatic hyperhomocysteinaemic patients suggests that many

would not consent to such a trial in view of the perceived safety and efficacy of vitamin therapy[4]. Second, we cannot be certain that the changes in endothelial function parameters were induced by the pyridoxine plus folic acid treatment, although this interpretation is supported by the strong correlation between post-treatment homocysteine and vWF levels (Figure 11.3). Nevertheless, the correlations observed between homocysteine levels and endothelial function parameters, although theoretically plausible, should be considered preliminary until confirmed in a larger group. As other cardiovascular risk factors did not change significantly during follow-up, changes in factors such as smoking habits are an unlikely explanation for the decreases in vWF and TM observed. Furthermore, vWF levels are relatively stable over periods of up to 3 years in (diabetic) patients remaining free of cardiovascular disease[13], suggesting that regression to the mean is also unlikely to explain the changes in vWF. Third, notwithstanding the promising effects of pyridoxine plus folic acid supplementation on endothelial function parameters, the clinical efficacy of the proposed treatment needs to be investigated in terms of prevention of new vascular events.

In conclusion, endothelial dysfunction is present in young patients with peripheral arterial occlusive disease and mHH. Pyridoxine plus folic acid treatment normalises homocysteine metabolism in the majority of patients, and appears to ameliorate endothelial dysfunction.

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References

1. Boers GHJ, Smals AGH, Trijbels JMF, Fowler B, Bakkeren JAJM, Schoonderwaldt HC, Kleijer WJ, Kloppenborg PWC. Heterozygosity for homocystinuria in premature peripheral and cerebral occlusive arterial disease. N Engl J Med 1985;313:709-715.
2. Clarke R, Daly L, Robinson K, Naughten E, Cahalane S, Fowler B, Graham I. Hyperhomocysteinemia: an independent risk factor for vascular disease. N Engl J Med 1991;324:1149-1155.
3. Mölgaard J, Malinow MR, Lassvik C, Holm AC, Upson B, Olsson AG. Hyperhomocyst(e)inaemia: an independent risk factor for intermittent claudication. J Intern Med 1992;231:272-279.
4. Ueland PM, Refsum H, Brattström L. Plasma homocysteine and cardiovascular disease. In: Francis RB (ed). Atherosclerotic cardiovascular disease, hemostasis, and endothelial dysfunction. Marcel Dekker Inc., New York, 1992;183-236.
5. Harker LA, Slichter SJ, Scott CR, Ross R. Homocystinemia. Vascular injury and arterial thrombosis. N Engl J Med 1974;291:537-543.
6. de Groot PG, Willems C, Boers GHJ, Gonsalves MD, van Aken WG, van Mourik JA. Endothelial cell dysfunction in homocystinuria. Eur J Clin Invest 1983;13:405-410.
7. Starkebaum G, Harlan JM. Endothelial cell injury due to copper-catalyzed hydrogen peroxide generation from homocysteine. J Clin Invest 1986;77:1370-1376.
8. Wang J, Dudman NPB, Wilcken DEL, Lynch JF. Homocysteine catabolism: levels of 3 enzymes in cultured human vascular endothelium and their relevance to vascular disease. Atherosclerosis 1992;97:97-106.
9. Stamler JS, Osborne JA, Jaraki A. Adverse vascular effects of homocysteine are modulated by endothelium-derived relaxing factor and related oxides of nitrogen. J Clin Invest 1993;91:308-318.
10. Brattström L, Israelsson B, Norrving B, Bergqvist D, Thörne J, Hultberg B, Hamfelt A. Impaired homocysteine metabolism in early-onset cerebral and peripheral occlusive arterial disease. Atherosclerosis 1990;81:51-60.
11. Suffredini AF, Harpel PC, Parrillo JE. Promotion and subsequent inhibition of plasminogen activation after administration of intravenous endotoxin to normal subjects. N Engl J Med 1989;320:1165-1172.
12. Jansson JH, Nilsson TK, Johnson O. von Willebrand factor in plasma: a novel risk factor for recurrent myocardial infarction and death. Br Heart J 1991;66:351-355.
13. Stehouwer CDA, Nauta JJP, Zeldenrust GC, Hackeng WHL, Donker AJM, den Ottolander GJH. Urinary albumin excretion, cardiovascular disease, and endothelial dysfunction in non-insulin-dependent diabetes mellitus. Lancet 1992;340:319-323.
14. Ishii H, Nakano M, Tsubouchi J, Ishikawa T, Uchiyama H, Hiraishi S, Tahara C, Miyajima Y, Kazama M. Establishment of enzyme immunoassay of human thrombomodulin in plasma and urine using monoclonal antibodies Thromb Haemostas 1990;63:157-162.
15. Ishii H, Uchiyama H, Kazama M. Soluble thrombomodulin antigen in conditioned medium is increased by damage of endothelial cells. Thromb Haemostas 1991;65:618-623.
16. Seigneur M, Dufourcq P, Conri C, Constans J, Mercié P, Pruvost A, Amiral J, Midy D, Baste J-C, Boisseau MR. Levels of plasma thrombomodulin are increased in atheromatous arterial disease. Thromb Res 1993;71:423-431.
17. Ridker PM, Vaughan DE, Stampfer MJ, Manson JE, Hennekens CH. Endogenous tissue-type plasminogen activator and risk of myocardial infarction. Lancet 1993;341:1165-1168.
18. Amiral J, Adam M, Mimilia F, Larrivaz I, Chambrette B, Boffa MC. A new assay for soluble forms of thrombomodulin in plasma. Thromb Haemostas

1991;65:947 (abstract).
19. Ubbink JB, Vermaak WJK, Bennett JM, Becker PJ, Staden DA, Bissbort S. The prevalence of homocysteinemia and hypercholesterolemia in angiographically defined coronary heart disease. Klin Wochenschr 1991;69:527-534.
20. Cejka J. Enzyme Immunoassay for Factor VIII-Related Antigen. Clin Chem 1982;28:1356-1358.
21. Ranby M, Bergsdorf N, Nilsson T, Mellbring G, Winblad B, Bucht G. Age dependence of tissue plasminogen activator concentrations in plasma, as studied by an improved enzyme-linked immunosorbent assay. Clin Chem 1986;32:2160-2165.
22. Kang SS, Wong PWK, Susmano A, Sora J, Norusis M, Ruggie N. Thermolabile methylenetetrahydrofolate reductase: An inherited risk factor for coronary artery disease. Am J Hum Genet 1991;48:536-545.
23. Stampfer MJ, Malinow MR, Willett WC, Newcomer LM, Upson B, Ullman D. A prospective study of plasma homocyst(e)ine and risk of myocardial infarction in US physicians. JAMA 1992;268:877-881.
24. Ross R. The pathogenesis of atherosclerosis: A perspective for the 1990s. Nature 1993;362:801-809.
25. Rodgers GM, Conn MT. Homocysteine, an atherogenic stimulus, reduces protein C activation by arterial and venous endothelial cells. Blood 1990;75:895-901.
26. Lentz SR, Sadler JE. Homocysteine inhibits von Willebrand factor processing and secretion by preventing transport from the endoplasmic reticulum. Blood 1993;81:683-689.
27. Lentz SR, Sadler JE. Inhibition of thrombomodulin surface expression and protein C activation by the thrombogenic agent homocysteine. J Clin Invest 1991;88:1906-1914.

IMAGING OF VASCULAR PATHOLOGY IN HYPERHOMOCYSTEINEMIC PATIENTS WITH DIGITAL SUBTRACTION ANGIOGRAPHY AND MAGNETIC RESONANCE TECHNIQUES

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Abstract

Mild hyperhomocysteinemia (mHH) is an independent risk factor for premature arteriosclerosis. We investigated the accuracy in the detection of early arteriosclerotic lesions in such patients. The left and right wall of the abdominal aorta, the aortic bifurcation and both common iliac arteries were evaluated with gated T1-weighted magnetic resonance imaging (gT1 MRI) and gated 2D-time of flight magnetic resonance angiography (g2D-TOF MRA) and were compared with intra-arterial digital subtraction angiography (iaDSA) in 11 patients with arteriosclerosis and mHH.

Six patients showed arteriosclerosis in one or more of the total number of 55 studied arterial segments with iaDSA. Thirty-two out of 37 normal and 12 out of 18 stenotic segments with gT1 MRI, and 29 normal and 6 stenotic segments with g2D-TOF MRA were correctly classified. Sensitivity of gT1 MRI and g2D-TOF MRA versus iaDSA was 67% and 33%, the specificity was 86% and 78%, and the accuracy was 80% and 64%, respectively.

We conclude that arteriosclerosis in patients with mHH is a regular finding, and gT1 MRI in such patients is an acceptable technique.

Introduction

Mild hyperhomocysteinemia (mHH) is an established risk factor for premature arteriosclerotic disease such as cerebral, peripheral and coronary arterial occlusive disease. Prevalence of mHH in vascular patients under 55 years of age is reported varying from 9% to 47% [1-3]. A recent multicenter study including more than 750 patients with arteriosclerosis and as much matched control subjects showed a relative risk of homocysteine levels in the upper decile of the distribution of control levels of about 4 to develop arterial occlusive disease [4]. The degree of peripheral arteriosclerosis might be related to the homocysteine concentration in blood [5] Very recently, the neuroradiological aspects of hyperhomocysteinemia has been extensively reported by van den Berg et al. [6].

Intraarterial digital subtraction angiography (iaDSA) has been accepted as the gold standard to detect the presence and to indicate the severity of arteriosclerosis in the vascular system. The main disadvantages of this technique are its invasive character and the involvement of ionizing radiation. The use of magnetic resonance imaging (MRI) and magnetic resonance angiography (MRA) has grown interest because these methods overcome these disadvantages. Furthermore, MRA can provide additional functional flow information. Several studies on MRI and MRA of the abdominal aorta and iliac arteries show a good correlation of the results of those techniques with those of conventional iaDSA [7-12]. In most of these studies the arteriosclerotic lesions were classified into mild stenosis (1 to 50% of arterial diameter), moderate stenosis (51% to 75%), or severe stenosis (76% to 100%). However, the accuracy and reliability of MRI and MRA examinations in the detection of mild arteriosclerotic occlusive disease has only poorly been evaluated. Proof of validity of these techniques is warranted before using these in the determination of early signs of generalized arterial disease in patients prone to arteriosclerosis A model of such generalized arteriosclerotic state is presented in subjects with elevated homocysteine levels in their blood, albeit mildly to moderately elevated concentrations compared to those in classical homocystinuria. Moreover, MRI and MRA studies in patients with mild hyperhomocysteinemia have not been reported until now [6].

Therefore, in this study we compared data obtained by means of MRI and MRA techniques with those provided by iaDSA in patients with proven arterial occlusive disease in the presence of mHH.

Methods

Patient Selection

In patients with premature arterial occlusive disease, mild hyperhomocysteinemia was established by means of a standardized methionine loading test measuring total homocysteine concentration in plasma [13-14] Combined supplementation of vitamin B6 and folic acid has proven to decrease and even to normalize pathological homocysteine blood levels in the majority of patients with arteriosclerosis and with mHH [15-16]. Clinically beneficial effect of such homocysteine-lowering intervention, in terms of decreasing number of vascular events, has not been demonstrated so far. Eleven detected hyperhomocysteinemic patients presenting post-load homocysteine levels above the 97.5 percentile in controls [16], seven men and four women, consecutively selected for participation in a running prospective placebo-controlled intervention study on the effect of

homocysteine-lowering treatment, were recruited for the present study. The eleven patients had suffered from arteriosclerotic disease as shown in Table 12.1. Cerebral arterial occlusive disease was confirmed by computer tomography. Coronary arterial occlusive disease was established by means of new Q waves on electrocardiography and/or diagnostic enzyme changes. Anterior spinal arterial syndrome was established by means of clinical presentation. This patient showed no infarction by means of computer tomography of the brain. Peripheral arterial occlusive disease was confirmed by an ankle-brachial blood pressure index less than 0.9. In one of these 2 patients, the ankle-brachial blood pressure measurements revealed arteriosclerotic changes in the superficial femoral artery at the right side and popliteal artery at the left side. In the other patient with peripheral arterial occlusive disease, the ankle-brachial blood pressure measurements revealed aortoiliac vascular changes. None of the patients revealed vitamin B_6, B_{12} or folic acid deficient blood levels, liver or renal disease, which conditions can also induce mHH [17]. Other risk factors for vascular disease such as diabetes, hyperlipoproteinemia and hypertension of non-renovascular origin were excluded. Not any of these patients was known to have a cardiac pace maker or surgical clips or was pregnant. The mean age ± 1SD of the patients was 41.6 ± 8.5 years (range 21 to 51 years) at the time of the examinations, and 38.9 ± 7.9 years at the time the vascular events had occurred (range 21 to 51 years). The protocol for this study had been approved by the Hospital Ethical Committee, and all included patients had signed an informed consent.

Table 12.1. The established vascular disease of which the patient suffered from is given with "cerebral" = cerebral arterial occlusive disease, "asas" = anterior spinal artery syndrome, "coronary" = coronary arterial occlusive disease, "peripheral" = peripheral arterial occlusive disease, age[1] ± SD = age at the time the first vascular events had occurred, and age[2] ± SD = age at the time of the examinations.

patient number	age[1] ± SD	age[2] ± SD	arteriosclerosis
1	37	40	cerebral
2	51	51	asas
3	41	45	cerebral
4	33	41	cerebral/coronary
5	41	51	cerebral/coronary
6	34	34	peripheral
7	45	46	cerebral/peripheral
8	45	46	coronary
9	41	42	cerebral
10	21	21	cerebral
11	39	40	cerebral
	38.9 ± 7.9	41.6 ± 8.5	

Intraarterial Digital Subtraction Angiography Technique

In this study, iaDSA was used as gold standard, to assess the reliability of MRI and MRA. IaDSA studies were performed with a Polytron 1000 VR (Siemens-Elema, Sweden) with a high resolution 1024x1024 pixel image matrix. Before the examination, all patients received orally 10 mg oxazepam (Seresta^R, Wyeth) and 0.25 mg atropin (Centrafarm) by intramuscular injection. Moreover, all patients received intravenously 20 mg of butylscopolaminebromide (Buscopan^R, Boehringer) as antiperistaltic medication directly before contrast medium injection. IaDSA was performed with a 5F pigtail catheter (Cook, the Netherlands) placed in the abdominal aorta at the level of the coeliac trunc by the transfemoral route. Twenty-five ml of meglumineamidotrizoaat 306 mg I/ml (Angiografin^R, Schering, the Netherlands) was diluted in ratio 1 to 1 with saline to which 37 5 IE heparin was added. Abdominal angiography in posteroanterior projection was obtained by injecting 50 ml diluted contrast media, with a flow-rate of 20 ml/s, an inject delay of 3.5 s and 3 frames per second.

Magnetic Resonance Imaging and Magnetic Resonance Angiography Technique

MRI and MRA examinations were performed with a 1.5 Tesla Magnetom 63 SP whole-body imaging system (Siemens Medical Systems, Erlangen, Germany). All patients received intravenously 20 mg of butylscopolamin (Buscopan^R) as antiperistaltic medication directly before and another 20 mg trough a continues drip infusion during the investigation. The abdomen was mildly compressed with a belt, and patients were allowed to breath normally during the examination. After scout images were obtained, the position of presaturation slices were placed superiorly to the image volume and over the anterior abdominal wall in order to suppress the signal from inflowing protons and respiratory and bowel motion artifacts. ECG triggered high resolution T1 weighted MR Images in coronal plane through the abdominal aorta and in axial angulated plane parallel to the common iliac arteries were acquired. The repetition time (TR) varied, depending the RR-interval, from 620 to 850 ms. Eleven slices were obtained using the following sequence parameters: echo time (TE) 25 ms, flip angle (α) 90^0, matrix size 315 x 512, field of view (FOV) 400 mm, contiguous slice thickness 4 mm, and 4 acquisitions. G2D-TOF MRA images were obtained in coronal plane with presaturation pulse placed inferiorly to the image volume and over the left side of the abdomen in order to suppress the signal from inflowing protons of the inferior cava vene and the left renal vein The following sequence parameters were used: 11 slices, TR 34 ms, TE 8 ms, matrix size 256 x 256, FOV 400 mm, contiguous slice thickness 4 mm, and 1 acquisition MIP reconstructions were made.

Evaluation

The period between MR and DSA examinations was less than 3 weeks in all patients. Two observers (JOB and FMJH) evaluated independently the MR and the DSA examinations, respectively, from hard copy films, without prior knowledge of each others results The evaluated abdominal aorta and the common iliac arteries of each patient were divided into 5 arterial segments, i.e. the left and right wall of the abdominal aorta, the aortic bifurcation, and the left and right common iliac artery, leading to a total of 55 evaluated segments in the 11 patients. Stenosis was graded according to the most severely narrowed part of the evaluated

segment compared with the luminal diameter of the normal appearing arterial segment in the vicinity of the arteriosclerotic plaque. The percentage of vessel diameter reduction was defined as:

$$100\% \times \frac{M1 - M2}{M1}$$

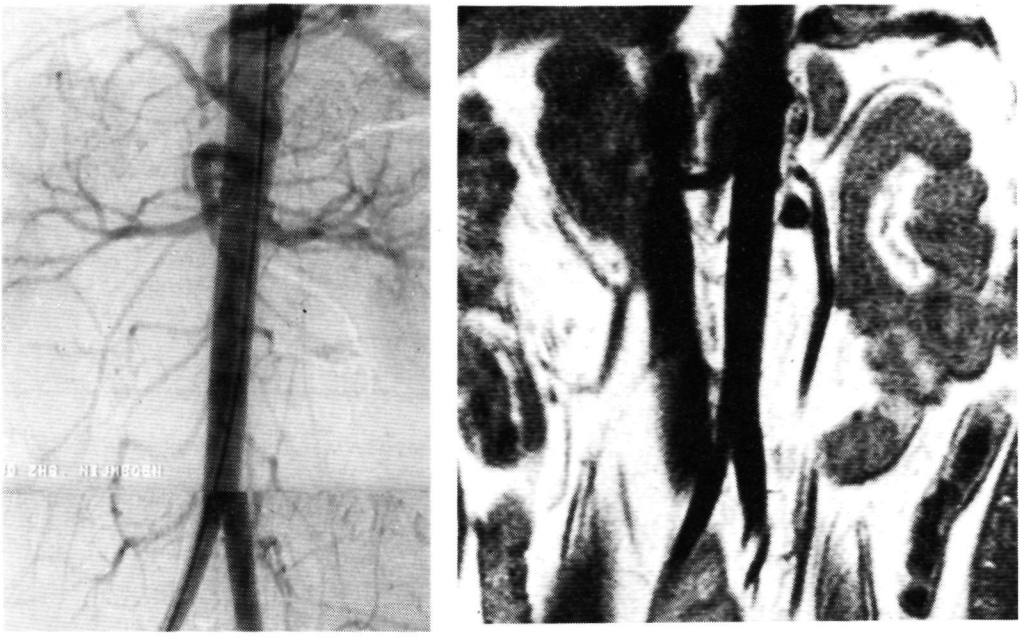
where M1 is the luminal diameter at the normal appearing level of the arterial segment; M2 is the luminal diameter of the arterial segment at the site of the most severe narrowing. In case of arteriosclerosis at both walls of the abdominal aorta, the percentage diameter narrowing was calculated for each side solely. The stenosis were grouped at intervals of 5% (e.g. 5, 10, 15 etc). The percentage diameter narrowing was subsequently divided into four categories: first, second, third and fourth degree arteriosclerosis with respectively 1% to 25%, 26% to 50%, 51% to 75%, 76% to 100% stenosis. In this study, the latter category was not observed.

Results

No complications occurred with the MRI and MRA examinations. One patient had a self-limiting catheter-induced hematoma after iaDSA examination at the punction side in the groin.

All arterial segments

Five out of all 11 patients were without arteriosclerosis, whereas the remaining 6 patients revealed arteriosclerosis in at least one evaluated arterial segment (Figures 12.1 and 12.2). In 18 out of 55 examined arterial segments, arteriosclerosis was detected by means of iaDSA showing narrowing varying from 5% to 70% of the luminal diameter (Tables 12.2 and 12.3). With gT1 MRI, 16 out of these 18 segments, and by g2D-TOF MRA, 7 out of 18 segments, were also classified as stenotic. Two and three out of 18 arteriosclerotic segments were classified incorrectly as normal by means of MRI and MRA, respectively. With the MRI technique, 3 segments with normal findings in iaDSA, and with MRA technique, 7 normal and 8 arteriosclerotic arterial segments in iaDSA could not be evaluated. The main reason was the loss of signal through which mostly the iliac arteries could not be depicted. The sensitivity for distinguishing normal and stenotic segments in the correct category with iaDSA as gold standard were higher by means of MRI than MRA examinations, i.e. 67% and 33% respectively. The specificity of correct evaluation of segments compared with iaDSA was 86% for MRI and 78% for MRA. Overall, the accuracy of gT1 MRI and g2D-TOF MRA evaluating arteriosclerosis in the abdominal aorta and the common iliac arteries compared to iaDSA in patients with arteriosclerosis and mHH was 80% and 64%, respectively. Three incorrectly evaluated segments by MRI were correctly evaluated by MRA. The sensitivity, specificity, and accuracy of combined MRI and MRA examinations was 72%, 92% and 85%, respectively.



a b

Figure 12.1. This 20 year old male patient was known with cerebral vascular disease leading to right sided hemiparesis and aphasia. He never smoked tobacco and had normal blood pressure measurements (125 mmHg and 85 mmHg, systolic and diastolic, respectively). The blood levels of glucose, cholesterol, triglycerides, creatinine, and liver enzymes (aspartate amino-transaminase and alanine amino-transaminase) were within normal ranges. His post-load plasma homocysteine level was 76 μmol/L (normal control value is below 55 μmol/L). The investigated arterial segments by means of IaDSA (Figure 12.1a) and gT1 MRI (Figure 12.1b) examinations proved to be without arteriosclerosis.

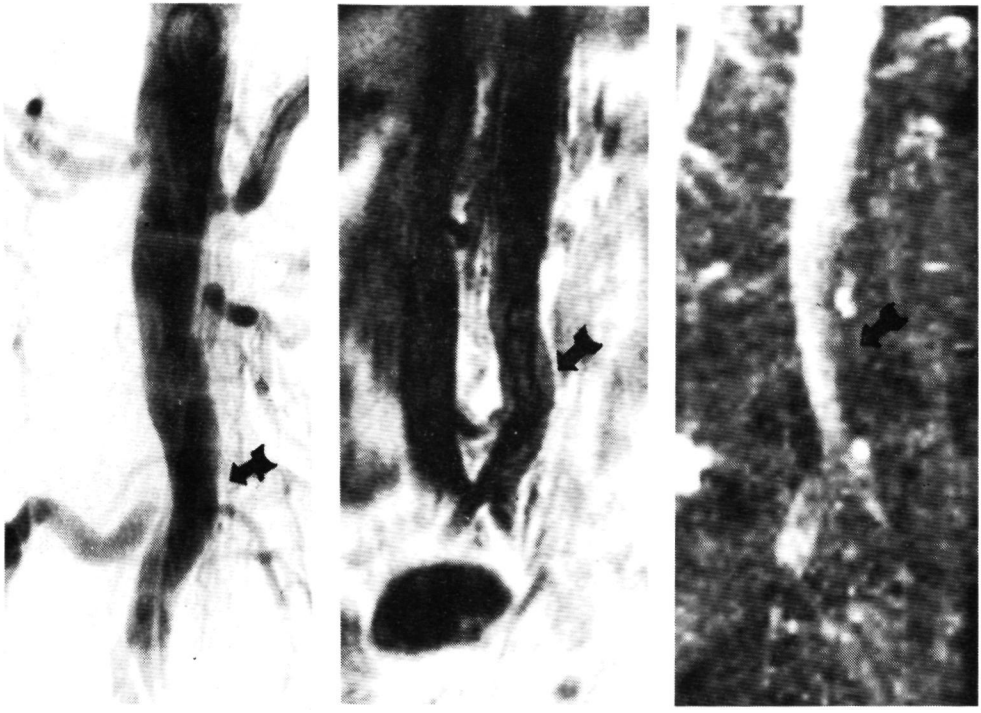


Figure 12.2. A 45 year old female patient who was known with cerebral vascular disease and intermittent claudication which first revealed several months before the intraarterial digital subtraction angiography (iaDSA; Figure 12.2a) and magnetic resonance imaging (MRI; Figure 12.2b) and magnetic resonance angiography (MRA; Figure 12.2c) were performed. She was known with 20 pack years of smoking and normal blood pressure measurements (130 mmHg and 85 mmHg, systolic and diastolic, respectively). The blood levels of protein C, antithrombin 3, glucose, cholesterol, triglycerides, creatinine, and liver enzymes (aspartate amino-transaminase and alanine amino-transaminase) were within normal ranges. Her post-load plasma homocysteine level was 127 μmol/L (normal control value is below 52 μmol/L). IaDSA (Figure 12.2a) and gT1 MRI (Figure 12.2b) revealed 30% stenosis at both sides of the abdominal aorta, 60% stenosis at the aortic bifurcation and the right common iliac artery, and 70% and 60% stenosis, respectively, at the left common iliac arterial segment.

Table 12 2. Findings of 55 arterial segments in 11 vascular patients with mild hyperhomocysteinemia evaluated with intraarterial digital subtraction angiography (iaDSA) and high resolution gated T1-weighted magnetic resonance imaging (MRI). Percentages are degree of vessel stenosis. With iaDSA, all vessel were evaluable; with MRI, 3 arterial segments were not evaluable (NE).

Degree of stenosis diagnosed with MRI	Degree of stenosis diagnosed with iaDSA 0%	1%-25%	26%-50%	51%-75%	76%-100%
NE	3	-	-	-	-
0%	32	2	-	-	-
1-25%	1	6	1	-	-
26%-50%	1	3	3	-	-
51%-75%	-	-	-	3	-
76%-100%	-	-	-	-	-
Total	37	11	4	3	0

Table 12.3. Findings of 55 arterial segments in 11 vascular patients with mild hyperhomocysteinemia evaluated with intraarterial digital subtraction angiography (iaDSA) and gated 2D-time of flight magnetic resonance angiography (MRA). Percentages are degree of vessel stenosis. With iaDSA, all vessel were evaluable; with MRA, 15 arterial segments were not evaluable (NE).

Degree of stenosis diagnosed with MRA	Degree of stenosis diagnosed with iaDSA 0%	1%-25%	26%-50%	51%-75%	76%-100%
NE	7	4	1	3	-
0%	29	3	-	-	-
1-25%	1	3	-	-	-
26%-50%	-	1	3	-	-
51%-75%	-	-	-	-	-
76%-100%	-	-	-	-	-
Total	37	11	4	3	0

The abdominal aorta

In 13 out of the 22 abdominal aortic segments, all three image modalities showed normal configuration of the arterial wall without arteriosclerosis, and 9 studied segments proved to be abnormal. Equivocal findings of the degree of arteriosclerotic lesions were determined with the MRI and MRA versus iaDSA in 7 and 5 segments, respectively. Consequently, MRI and MRA were classifying incorrectly in 2 and 4 segments. Slight arteriosclerosis by means of iaDSA was underestimated and classified as normal in two segments with MRI and in three segments with MRA In one segment with first degree vascular changes, MRA examination was overestimated showing second degree arteriosclerosis.

Aortic bifurcation

In 7 out of 11 segments the three image modalities revealed no arteriosclerosis at the site of the aortic bifurcation. In the 4 other patients, the aortic bifurcation proved to have vascular damages by means of the golden standard iaDSA. One arteriosclerotic aortic bifurcation was correctly identified with all examinations. MRI overestimated the arteriosclerosis in two arterial segments and underestimated the abnormality in one segment. No abnormal aortic bifurcation was classified as normal with the MRI technique. With MRA, the aortic bifurcation in two patients could not be evaluated on the basis of signal loss, and in one patient the degree of arteriosclerosis was overestimated as slight arteriosclerotic vascular changes in stead of normal vascular arterial wall.

Common iliac arteries

Three out of the 22 segments with MRI and 13 segments with MRA could not be evaluated due to different angulation than the image plane, and due to spin saturation, respectively. IaDSA and MRI showed equal findings in 12 normal segments and in 4 stenotic segments. MRI overestimated in three segments, i.e. one normal vascular wall was classified as second degree arteriosclerosis, and two first degree arteriosclerotic changes were classified as second degree arteriosclerosis Nine normal iliacal segments were correctly assessed with MRA examination.

Discussion

Mild hyperhomocysteinemia is established as a condition which leads to generalized arteriosclerosis with or without vascular symptoms [5,18]. In this study, indeed 6 out of all 11 patients (55%) revealed arteriosclerosis established by means of iaDSA in at least one of the 5 evaluated arterial segments in each patient. In only one patient the demonstrated vascular damage was leading to clinical symptoms and was confirmed by an abnormal ankle-brachial blood pressure index. The other five patients were without clinical symptoms despite their percentage of vessel diameter narrowing upto 30%.

The gT1 MRI technique determined 12 out of 18 stenotic segments, and 32 out of 37 normal segments correctly. The 6 incorrectly evaluated stenotic segments were overestimated in three and underestimated also in three. All incorrectly classified arterial segments showed a discrepancy of only one degree, except one normal iliac segment which showed second degree arteriosclerosis by means of gT1 MRI. The mean (± SD) percentage of discongruence between MRI and DSA measurements proved to be 10% (± 5%) of vessel diameter. The sensitivity of correctly cathegorizing by gT1 MRI versus iaDSA was 67%, the specificity was 86%, and the accuracy was 80%. Therefore, we conclude that high resolution gT1 MRI of the abdominal aorta, aortic bifurcation, and the common iliac arteries in patients with arteriosclerosis and mild hyperhomocysteinemia appears to be an acceptable imaging method in terms of accuracy compared with iaDSA, and may be an alternative method to the invasive iaDSA technique

By means of MRA examination, only 6 out of 18 stenotic segments and 29 out of 37 normal segments were classified correctly One stenotic segment by means of iaDSA was overestimated with MRA and 3 slightly stenotic segments were classified as normal. We were unable to classify 15 arterial segments with MRA mainly based on signal loss at the level of the bifurcation or more distally

The reduced visualization of flowing blood in the more distal imaged volume with MRA, is related to the progressive spin saturation leading to signal loss. The high proportion of not evaluable examinations with the g2D-TOF MRA technique is the main reason for the accuracy as low as 64% of MRA compared with iaDSA in this study. MRA was classifying correctly compared to iaDSA in contrast to a misdiagnose by MRI, in only two out of the 55 segments. Therefore, MRA does not seems to provide significant additional information to gT1 MRI. Further studies may disclose improvement of newer MRA techniques in their determination of arteriosclerosis in abdominal and pelvic arteries.

To generate signal with gT1 MRI, protons must remain for at least ½ TE time interval to be exposed Blood flow, remaining shorter than ½ TE time during the exposure time, produces signal void. The reduced signal from flowing blood compared with the signal from arterial walls and vessel wall damages provides excellent contrast for detection of arteriosclerosis. However, blood flow velocity is highest in the center of an artery and slower at the vessel wall and turbulance distal to the arteriosclerotic lesion may lead to overestimation of the degree of arteriosclerosis with gT1 MRI [19], occuring in 5 out of 55 segments in this study. GT1 MRI was underestimated in 3 out of 55 segments, all three on the base of only 5% stenosis difference assessed by iaDSA and MRI.

Maximum enhancement with g2D-TOF MRA occurs when the velocity of blood is such high that the initially exitated blood volume is completely replaced by "fresh" inflowing blood spins in the imaged volume. A disadvantage of g2D-TOF MRA method appears when the imaged volume is too large to be fully replaced and this is the reason that 15 arterial segments, 2 aortic bifurcations and 13 iliac arterial segments, were not evaluable and 2 segments were overestimated with the g2D-TOF MRA technique in this study. A thinner slice tickness could increase the signal intensity, but consequently the examination time would increase unacceptably and the signal to noise ratio will decrease.

Conventional angiography is nowadays a well accepted technique, but remains nevertheless not completely without risk Renal dysfunction, embolism and puncture site trauma, such as hematoma, bleeding, pseudoaneurysm, dissection, arteriovenous fistula, thrombosis, and infection are the main complications Furthermore, iaDSA can provide only two-dimensional images [20].

MR images are performed without vessel invasion which is attractive especially in arteriosclerotic patients. One of the further merits of MRI technique, in contrast to iaDSA, is that it provides also information of surrounding tissues.

Although the number of patients is limited, these initial results show, that high resolution gated T1-weighted MRI is of such image quality that it is a acceptable alternative to conventional angiography to determine generalized though mild arteriosclerotic arterial damages such as in patients with mild hyperhomocysteinemia In the follow up of the clinical effect of homocysteine-lowering treatment in such patients this non-invasive technique is preferable. Gated two dimensional time of flight MRA does not provide additional information to gT1 MRI.

References

1. Boers GHJ, Smals AGH, Trijbels JMF, Fowler B, Bakkeren JAJM, Schoonderwaldt HC, Kleijer WJ, Kloppenborg PWC. Heterozygosity for homocystinuria in premature peripheral and cerebral occlusive arterial disease. N Engl J Med 1985;313:709-715.
2. Kang S-S, Wong PWK, Malinow MR. Hyperhomocyst(e)inemia as a risk factor for occlusive vascular disease. Annu Rev Nutr 1992;12:279-298.
3. Ueland PM, Refsum H, Brattstrom L. Plasma homocysteine and cardiovascular disease. In: Francis RBF (ed). Atherosclerotic cardiovascular disease, hemostasis, and endothelial function. Marcel Dekker Inc., New York, 1992;183-236.
4. Graham I, Daly L, Ueland PM, Robinson K, Brattstrom L, Refsum H, et al. Homocysteine and vascular disease: The European concerted action project. Submitted 1996.
5. van den Berg M, Stehouwer CDA, Bierdrager E, Rauwerda JA. Plasma homocysteine and severity of atherosclerosis in young patients with lower extremity atherosclerotic disease. Arterioscler Thromb Vasc Biol 1996,16:165-171.
6. van den Berg M, van der Knaap MS, Boers GHJ, Stehouwer CDA, Rauwerda JA, Valk J. Hyperhomocysteinemia: with reference to its neuroradiological aspects. Neuroradiology 1995;37:403.
7. Mulligan SA, Doyle M, Matsuda T, Koslin DB, Kenney PJ, Barton RE, Pohost CM Aortoiliac disease: two-dimensional inflow MR Angiography with lipid suppression. JMRI 1993;3:829-834
8. Krug B, Kugel H, Harnischmacher U, Heindel W, Fischbach R, Altenburg A, Krings F Diagnostic performance of digital subtraction angiography (DSA) and magnetic resonance angiography (MRA): preliminary results in vascular occlusive disease of the abdominal and lower-extremity arteries. Eur J of Radiol 1995;19:77-85.
9. Arlart IP, Guhl L, Edelman RR. Magnetic resonance angiography of the abdominal aorta. Cardiovasc Intervent Radiol 1992,15:43-50.
10. Kim D, Edelman RR, Kent KC, Porter DH, Skillman JJ. Abdominal aorta and renal artery stenosis: evaluation with MR Angiography. Radiology 1990;174:727-731.
11. Yoshikawa K, Sugimura K, Kawamitsu H, Ishida T. Intrapelvic two-dimensional time-of-flight magnetic resonance angiography in healthy and diseased subjects. Brit J of Radiol 1994;67:140-146.
12. Cambria RP, Yucel EK, Brewster DC, L'Italien G, Gertler JP, LaMuraglia GM, Kaufman JA, Waltman AC, Abbott WM. The potential for lower extremity revascularization without contrast arteriography: Experience with magnetic resonance angiography. J Vasc Surg 1993;17:1050-1057.
13. Boers GHJ, Smals AGH, Trijbels JMF, Leermakers AI, Kloppenborg PWC. Unique efficiency of methionine metabolism in premenopausal women may protect against vascular disease in the reproductive years. J Clin Invest 1983;72.1971-1976.
14. te Poele-Pothoff MTWB, van den Berg M, Franken DG, Boers GHJ, Jakobs C, de Kroon IFI, Eskes TKAB, Trijbels JMF, Blom HJ. Three different methods for the determination of total homocysteine in plasma Ann of Clin Biochem 1995;32:218-220.
15. Franken DG, Boers GHJ, Blom HJ, Trijbels JMF, Kloppenborg PWC. Treatment of mild hyperhomocysteinemia in vascular disease patients. Arterioscler Thromb 1994;14:465-470
16. van den Berg M, Franken DG, Boers GHJ, Blom HJ, Jakobs C, Rauwerda JA. Combined vitamin B6 plus folic acid therapy in young patients with arteriosclerosis and hyperhomocysteinemia. J Vasc Surg 1994;20:933-940.
17. Ueland PM, Refsum H Plasma homocysteine, a risk factor for vascular disease Plasma levels in health, disease, and drug therapy. J Lab Clin Med

1989;114:473-501.
18. Malinow MR, Nieto FJ, Szklo M, Chambless LE, Bond G. Carotid artery intimal-medial wall thickening and plasma homocyst(e)ine in asymptomatic adults. Circ 1993;87:1107-1113.
19. von Schulthess GK. Blood flow. In: Higgings CB, Hricak H, Helms CA (eds) Magnetic Resonance Imaging of the body. Raven Press, New York, 1992;313-337.
20. Waugh JR, Sacharias N. Arteriographic complications in the DSA era. Radiology 1992;182:243.

IS HOMOCYSTEINE-LOWERING TREATMENT BENEFICIAL IN ARTERIOSCLEROSIS DUE TO MILD HYPERHOMOCYSTEINEMIA?

Preliminary results of a prospective trial.

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Submitted.

Abstract

Supplementation of combined vitamin B_6 and folic acid can normalize elevated homocysteine blood levels, a risk factor for arteriosclerosis and thrombosis. Clinically beneficial effect of this treatment by means of slowing down the progress of arteriosclerosis has not been established until now. A 2 year running double-blind placebo-controlled prospective trial in vascular patients with mild hyperhomocysteinemia started, to assess the effect of this treatment. Evaluation of arteriosclerosis is performed with Magnetic Resonance Imaging of the abdominal aorta and proximal segments of the common iliac arteries, segmental blood pressure measurements of the lower extremities and duplex scanning of the carotid and vertebral arteries.

In this preliminary report on the outcome in a small group of the first 14 included vascular hyperhomocysteinemic patients, it is shown that 8 had also arteriosclerotic signs at segments where their clinical symptoms did not originate from. Four out of 8 vitamin-treated and one of the 6 placebo-treated patients showed regression, and 3 placebo-treated patients showed progression of arteriosclerosis, in contrast to one in the vitamin-treated group. In the remaining 5 patients, 3 vitamin and 2 placebo-treated, the arteriosclerosis was overall unchanged.

We conclude from this preliminary trial that in vascular patients with mild hyperhomocysteinemia, arteriosclerosis is found to be very often generalized. Although these results are small in number, they do not contradict a possible potency of combined vitamin B_6 and folic acid to prevent progression of arteriosclerotic changes in vascular patients with mild hyperhomocysteinemia.

Introduction

With a frequency which varies from 9% to 47% of patients with premature arteriosclerosis leading to occlusive cerebral, peripheral, or coronary artery disease, mild hyperhomocysteinemia (mHH) has been detected retrospectively in numerous studies[1]. The association between premature arteriosclerosis and mHH has also been found in large epidemiological prospective studies[2-4].

Until now, two enzyme deficiencies have been detected in vascular patients which may lead to mildly elevated blood homocysteine levels, i.e. cystathionine synthase[5,6] and thermolabile 5,10-methylenetetrahydrofolate reductase deficiency[7-11]. The former enzyme requires pyridoxal 5'-phosphate, the biologic active form of vitamin B_6, as cofactor. The latter enzyme catalyzes 5,10-methylenetetrahydrofolate into 5-methyltetrahydrofolate, which is the main form of folate in blood. Vitamin B_6 supplementation solely, in a dose of 250 mg daily, lowers in 76%, and even normalizes in 56% of vascular patients the elevated post-load homocysteine concentrations[12]. Combined supplemention of vitamin B_6 and folic acid normalizes the pathological homocysteine blood levels in the vast majority of hyperhomocysteinemic patients who poorly or not at all respond to sole vitamin B_6 treatment[12,13]. This combined homocysteine-lowering treatment even improves some markers of endothelial dysfunction in vascular patients with mHH[14]. Reportedly, the incidence of vascular accidents is significantly reduced after initiating homocysteine-lowering treatment in severe hyperhomocysteinemia, revealing the clinically beneficial effect of such intervention[15]. However, it is not clarified whether homocysteine-lowering treatment has also such positive effect in vascular patients with mHH in terms of a regression or stabilization of arteriosclerosis. Hence, we have started a 2 year running double-blind placebo-controlled prospective study with combined vitamin B_6 and folic acid treatment in vascular patients with mHH. Evaluation of the arteriosclerotic lesions was performed with Magnetic Resonance Imaging (MRI) of the abdominal aorta and proximal segments of the common iliac arteries, segmental blood pressure (SBM) and duplex measurements (DU) of the carotid arteries. The results of outcome after 2 years of inter-vention in the first 14 treated patients are presented here preliminarily.

Materials and methods

Patients

Patients were selected consecutively among those suffering from arterial vascular disease which had become symptomatic before the age of 55 years who showed after methionine loading elevated homocysteine concentration. Conventional risk factors such as diabetes, hypercholesterolemia and hypertension unless of renovascular origin were excluded, however, cigarette smoking (range from 0 to 26 pack years of smoking, one pack year is defined as smoking one pack of cigarettes daily for one year) was not. Of the first 14 of such patients, seven patients had suffered from stroke due to cerebral vascular disease (CVD), two from coronary heart disease (CHD), one from intermittent claudication due to peripheral vascular disease (PVD), one from anterior spinal arterial syndrome (SAS), two from combined CVD and CHD, and one from combined CVD and PVD. The clinical diagnosis in patients with stroke was confirmed by computer tomography, and in patients with PVD by SBM. All patients with CHD had undergone a bypass operation or percutaneous transluminal coronary angioplasty. The patient with SAS

presented with loss of sensibility and strength below the level of thoracal VIII at the right, and thoracal IX at the left side, and with paresis of the urine bladder. In this patient, the diagnosis SAS was based on clinical presentation The vascular disease in the patients became symptomatic between their 20th and 50th year of age (37 ± 6 years; mean ± SD). The age of the 14 patients at the start of the study was 39 ± 7 years (mean age ± SD). Not any of these patients were known to have a cardiac pace maker, metallic surgical clips or was pregnant in an early stage as these factors are a contraindication for MRI examination

Trial design

All patients were known with post-load elevated homocysteine levels, which decreased within the range of normal controls after combined vitamin B_6 and folic acid treatment, 250 mg and 5 mg daily, respectively Although we have had reported previously that this combined treatment will normalize the post-load homocysteine levels in almost all hyperhomocysteinemic vascular patients[13], all the studied patients had to prove such normalization after combined homocysteine-lowering treatment during 6 weeks after a repeated methionine loading test, for reasons of scrutiny, before entering this prospective study Informed consent was obtained from each patient. The patients were randomized in a double-blind way into either a vitamin (i.e. vitamin B_6, 250 mg daily, and folic acid, 5 mg daily) or a placebo-treated group. At study entry, one and two years after the start of the study, all patients underwent a methionine loading test, SBM, DU, standardized questionnaire, physical examination, and electrocardiography in supine position. Standardized questionnaire was focused upon complaints of arterial ischaemia, and physical examination was carried out, with special attention to arterial pulsations in neck and extremities MRI was performed at study entry and after two years

The research protocol for this study had been approved by the Hospital's Ethical Committee

Methionine loading test

MHH was established by means of increased total homocysteine blood concentrations above mean control level plus 2 standard deviations after a standardized methionine loading, as described earlier[16] Venously collected plasma samples in the fasting state and 6 hours after methionine loading (0.1 g L-methionine/kg body weight) were centrifuged instantly and total homocysteine concentrations (free plus protein bound) were measured by high-performance liquid chromatography[17] Because the mean post-load homocysteine levels between control men, pre-, and postmenopausal women differs, the studied patients were categorized accordingly[5 16 18 19]. Moreover, fasting vitamin B_6 (normal range: 28 - 107 nmol/L), vitamin B_{12} (160 - 750 pmol/L), folic acid (5 5 - 40 0 nmol/L), creatinine, and liver enzymes (aspartate amino-transaminase and alanine amino-transaminase) levels were measured by means of standard procedures.

Segmental blood pressure measurement (SBM)

Pressure measurements were performed by means of 4 pair of cuffs strapped around the lower extremities, i e at high thigh, above the knee, below the knee, and at ankle levels[20]. Pressure measurements were determined at all levels, with a pocket Doppler apparatus (Omex, USA) The highest systolic brachial artery

pressure was used as a reference for the systemic blood pressure. Measurements were performed at rest and during reactive hyperaemia induced by a 5 minutes arterial occlusion, by means of thigh cuff compression with pressures above the systolic arterial pressure with the patient in supine position. When the leg-brachial arterial pressure index was beneath 0.90 at rest, or beneath 0.85 after 5 minutes occlusion, the SBM was considered abnormal. If after one or two years one of these indices had passed these values or had changed by more than 0.2, this was considered as a significant alteration in arterial occlusive disease.

Duplex scanning measurements (DU)

Blood flow velocity measurements of extracranial part of the internal, external, and the common carotid arteries, and vertebral arteries at both sides was performed by means of duplex sonographic scanning. The velocity of the internal carotid arteries was performed at 3 levels. Thus, measurements at 6 levels are determined at each side of the patient The patient was in supine position with the neck flexed contralateral to the examined side. A Toshiba SSA-270 A system was used, equipped with 7 5-Mhz linear probe which was positioned parallel to each arterial segment for color Doppler imaging. The blood velocity during the early systolic peak and during the diastolic phase were measured The grade of stenosis was scored according Strandness[21]. In short: stenosis up to 49% was defined when peak systolic velocity did not raise above 1.25 m/s. Velocities above this blood flow level indicates stenosis of 50% or more Furthermore, stenosis of 50% to 75% was defined when peak systolic velocity ratio (i.e. peak systolic velocity divided by peak systolic velocity proximal of this level) was ≥ 2 and < 3, and stenosis of 76% to 99% was determined when peak systolic velocity ratio raised above 3. Occluded arterial segments showed no blood flow.

Magnetic Resonance Imaging (MRI)

MRI of the abdominal aorta and proximal segments of the common iliac arteries was performed with 1.5 Tesla Magnetom 63 SP whole-body imaging system (Siemens, Germany), according to a protocol reported previously[22]. Briefly, all patients received intravenously 20 mg of butylscopolamin (Buscopan^{R}) as antiperistaltic medication directly before and another 20 mg via a continuous infusion during the investigation. High resolution T1-weighted MRI in coronal (frontal) and sagittal (lateral) direction of the abdominal aorta and axial angulated orientated images parallel to the common iliac arteries were obtained during ECG gating. The percentage diameter narrowing was measured and grouped at intervals of 5% (e.g 5, 10, 15 etc). To determine the accuracy of MRI, we previously have performed a digital subtraction angiography as golden standard in 11 out of 14 patients at the start of the study[22]. The accuracy of high resolution MRI versus iaDSA was 80%. From that study, it is to conclude that this MRI technique is an acceptable technique for determination of arteriosclerosis in hyperhomocysteinemic vascular patients. The percentage of vessel diameter reduction was defined as:

$$100\% \times \frac{M1 - M2}{M1}$$

where M1 is the luminal diameter at the normal appearing level of the arterial segment; M2 is the luminal diameter of the arterial segment at the site of the most severe narrowing.

Statistics

If arteriosclerosis determined by the various examination techniques used in this trial led to the establishment of progression and regression within one patient, the overall conclusion was defined as progression. The effect of treatment in the studied vascular patients with mHH were analyzed by a chi-square independence test. To determine significant difference in vitamin B_6, folic acid, vitamin B_{12}, fasting and post-load homocysteine blood concentrations of the vitamin-treated and placebo-treated group of patients a analyses of variance (ANOVA test) has been used.

Results

Asymptomatic arteriosclerosis

At the start of the study, 8 out of 14 patients (57%) showed arteriosclerotic changes in arterial segments from which no clinical symptoms originated (Table 13.1; patient number 1, 2, 4, 5, 8, 10, 11, and 14). Four out of these 8 patients have had CVD, one patient has had CHD, one has had SAS, and 2 patients have had combined CVD and CHD. Arteriosclerotic damages were determined by means of SBM in 7 out of these 8 patients, and by MRI in 5 patients.

Biochemical effect of treatment

In all included patients the levels of creatinine and liver enzymes remained within normal ranges throughout the study period. Nine out of the 14 patients with post-load hyperhomocysteinemia were also known with elevated fasting homocysteine levels in plasma. At baseline, the mean fasting and post-load homocysteine blood levels were 26 ± 22 μmol/L (mean ± SD) and 83 ± 26 μmol/L, respectively, in the group of patients randomized for vitamin treatment, not significantly different from those in patients randomized for placebo treatment, i.e. 27 ± 13 μmol/L and 79 ± 26 μmol/L, respectively (Table 13.2). In all 14 patients the mean fasting and post-load homocysteine blood levels could be normalized after probational vitamin treatment for 6 weeks, showing then a mean of 10 ± 3 μmol/L (mean ± SD) and 39 ± 7 μmol/L, respectively, with no significant difference of the homocysteine concentrations between the vitamin and placebo-treated group. The mean reduction of fasting and post-load homocysteine concentrations after this combination of vitamin supplementation was 74% and 68%, respectively. Also the blood levels of vitamin B_6, vitamin B_{12}, and folic acid were at baseline and after probational treatment not significantly different between the 2 groups.

Vitamin B_6, folic acid, vitamin B_{12}, fasting and post-load homocysteine blood concentrations after one and two years of treatment were not significantly different from the levels at baseline in the placebo-treated patients. During this period of follow-up not any of the vitamin-treated patients became hyperhomocysteinemic again, and all of the placebo-treated patients continued to have pathologically elevated homocysteine blood levels.

Table 13.1. Evaluation of arteriosclerosis at baseline and after two years of vitamin (patient number 1 to 8) or placebo (patient number 9 to 14) supplementation was performed with segmental blood pressure measurements (SBM) of the lower extremities, duplex scanning (DU) of the carotid and vertebral arteries, and Magnetic Resonance Imaging (MRI) of the abdominal aorta and proximal segments of the common iliac arteries. Patient number 8 received the first year placebo and the second year vitamin supplementation. With SBM and DU, the abnormal side, and with MRI, the percentage of occlusion in the segment in which the vascular damage had changed after two years are given in this table. n = normal; R = right side abnormal; L = left side abnormal. ↑ = SBM improved by more than 0.20. ↓ = SBM deteriorated by more than 0.20.

Patient number		Baseline	After 2 years
1.CVD	SBM	R & L	n
	DU	n	n
	MRI	20%	10%
2.SAS	SBM	L	n
	DU	n	n
	MRI	n	n
3.PVD	SBM	R & L	R & L↑
	DU	n	n
	MRI	30%	30%
4.CVD	SBM	R & L	n
	DU	n	n
	MRI	5%	5%
5.CVD&CHD	SBM	L	n
	DU	n	R & L
	MRI	30%	30%
6.CHD	SBM	n	n
	DU	n	n
	MRI	n	n
7.CVD	SBM	n	n
	DU	n	n
	MRI	n	n
8.CHD	SBM	n	n
	DU	n	n
	MRI	20%	20%
9.CVD&CHD	SBM	R & L	R↓ & L
	DU	n	n
	MRI	5%	25%
10.CVD&PVD	SBM	R & L	R↓ & L↓
	DU	n	n
	MRI	60%	100%
11.CVD	SBM	R & L	n
	DU	R 50 - 75%	R 100%
	MRI	n	n
12.CVD	SBM	R & L	L at rest
	DU	n	n
	MRI	n	n
13.CVD	SBM	n	n
	DU	n	n
	MRI	n	n
14.CVD	SBM	n	n
	DU	n	n
	MRI	n	n

Table 13.2. Mean ± SD values are shown for the continuous variables, in all patients (n = 14), thus included the patient with one year placebo, and one year vitamin therapy, for the vitamin-treated (n = 8) and placebo-treated (n = 6) patients. Probational therapy consisted of 250 mg vitamin B_6 and 5 mg folic acid daily for 6 weeks, which continued in the 8 vitamin-treated patients for 2 years, and was withheld from in the placebo-treated group of patients. Statistically significance was calculated with ANOVA in the vitamin-treated group versus the placebo-treated group of patients * < 0.05; # < 0.01.

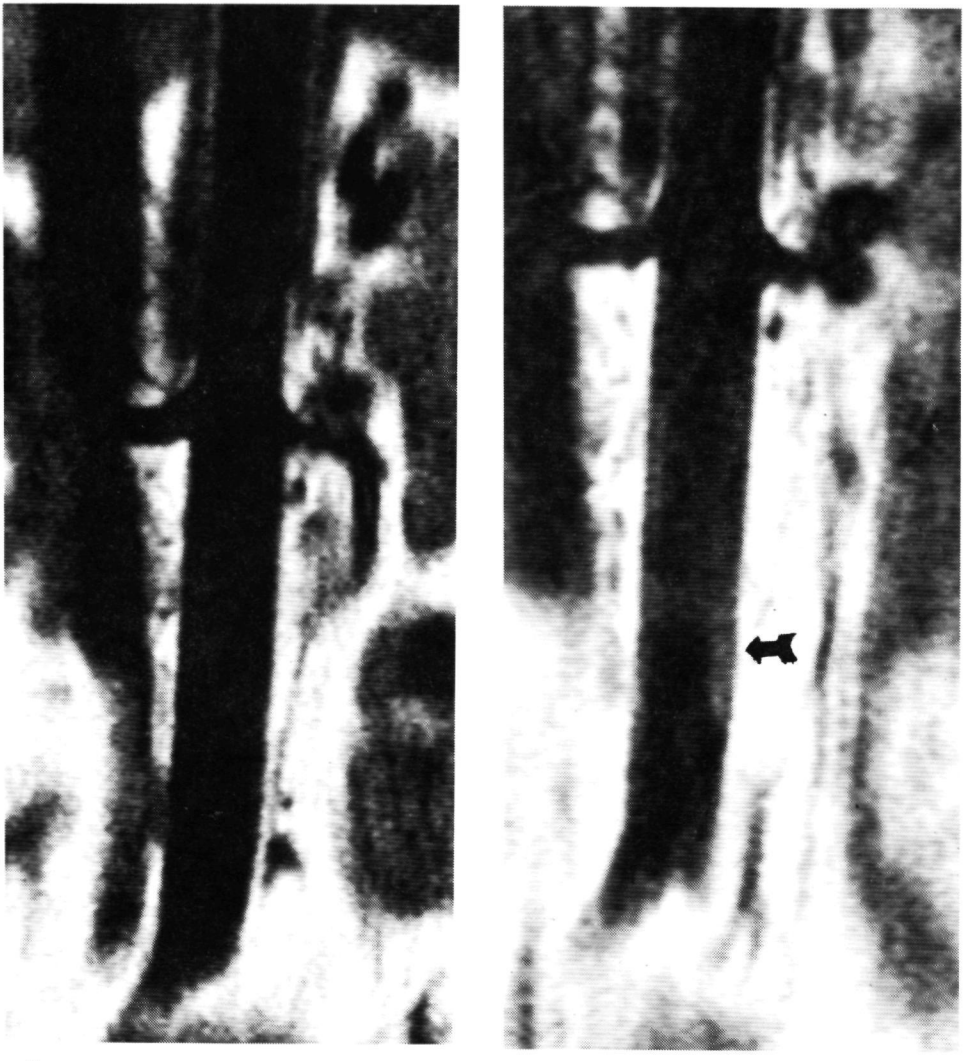
	vitamin-treated patients (n = 8)	all patients (n = 14)	placebo-treated patients (n = 6)
fasting homocysteine μmol/L			
baseline level	26 ± 22	26 ± 17	27 ± 13
after probational therapy	11 ± 2	10 ± 3	10 ± 3
after one year*	11 ± 4		24 ± 12
after two years#	12 ± 3		25 ± 10
post-load homocysteine μmol/L			
baseline level	83 ± 26	80 ± 24	79 ± 26
after probational therapy	40 ± 9	39 ± 7	40 ± 6
after one year*	41 ± 8		71 ± 28
after two years*	44 ± 14		73 ± 23
vitamin B_6 nmol/L			
baseline level	52 ± 7	54 ± 19	60 ± 27
after probational therapy	505 ± 223	871 ± 1343	1380 ± 2020
after one year*	2271 ± 2192		64 ± 28
after two years	1885 ± 2144		51 ± 15
folic acid nmol/L			
baseline level	9.9 ± 2.5	9.8 ± 2.7	9.5 ± 3.2
after probational therapy	72.1 ± 36.7	77.1 ± 32.5	77.5 ± 29.6
after one year*	182.0 ± 178.4		8.7 ± 3.8
after two years	206.7 ± 232.1		7.1 ± 3.4
vitamin B_{12} pmol/L			
baseline level	256 ± 145	300 ± 210	372 ± 280
after probational therapy	231 ± 33	221 ± 35	215 ± 9
after one year	200 ± 64	255 ± 220	332 ± 330
after two years	361 ± 416	273 ± 312	164 ± 45

Clinical effect of treatment

One patient had erroneously received the first year placebo and the second year vitamin supplementation, which was confirmed by normal respectively elevated levels of these vitamins in blood, and elevated respectively normal fasting and afterload homocysteine concentrations. In that patient, who had suffered from coronary heart disease with no vascular changes at the studied segments after both the first and the second year, neither progression or regression was concluded (Table 13.1, patient number 8). Eight patients, included the above mentioned, appeared retrospectively to be treated with vitamin, and 6 patients with placebo supplementation. All randomized patients completed this prospective study for 2 years. Although cigarette smoking was discouraged, 3 patients continued their smoking habit after the vascular disease revealed. At the start of the study, there was no difference in the mean pack years of smoking between the vitamin and placebo-treated patients, i.e. 12 and 13, respectively.

Four out of 8 vitamin-treated patients showed regression of the arteriosclerosis as assessed in all 4 with SBM (Table 13.1; patient number 1, 2, 3, and 4). In these four patients, the blood pressure of the lower extremities in two legs in rest and five legs after occlusion increased significantly after 2 years. Additionally, with MRI in one of these four patients, the initial 20% stenosis at the abdominal aorta showed a decrease to 10% after two years. In the other 3 of these 4 patients, MRI and DU findings were not changed. In one other vitamin-treated patient (Table 13.1; patient number 5) the aberrant SBM at rest of the left lower extremity improved significantly, whereas DU showed accelerated blood flow velocity in both external carotid arteries not at baseline, but after two years. This was defined as progression of arteriosclerosis. In the resulting other 3 vitamin-treated patients the arteriosclerosis remained unchanged (Table 13.1; patient number 6, 7 and 8).

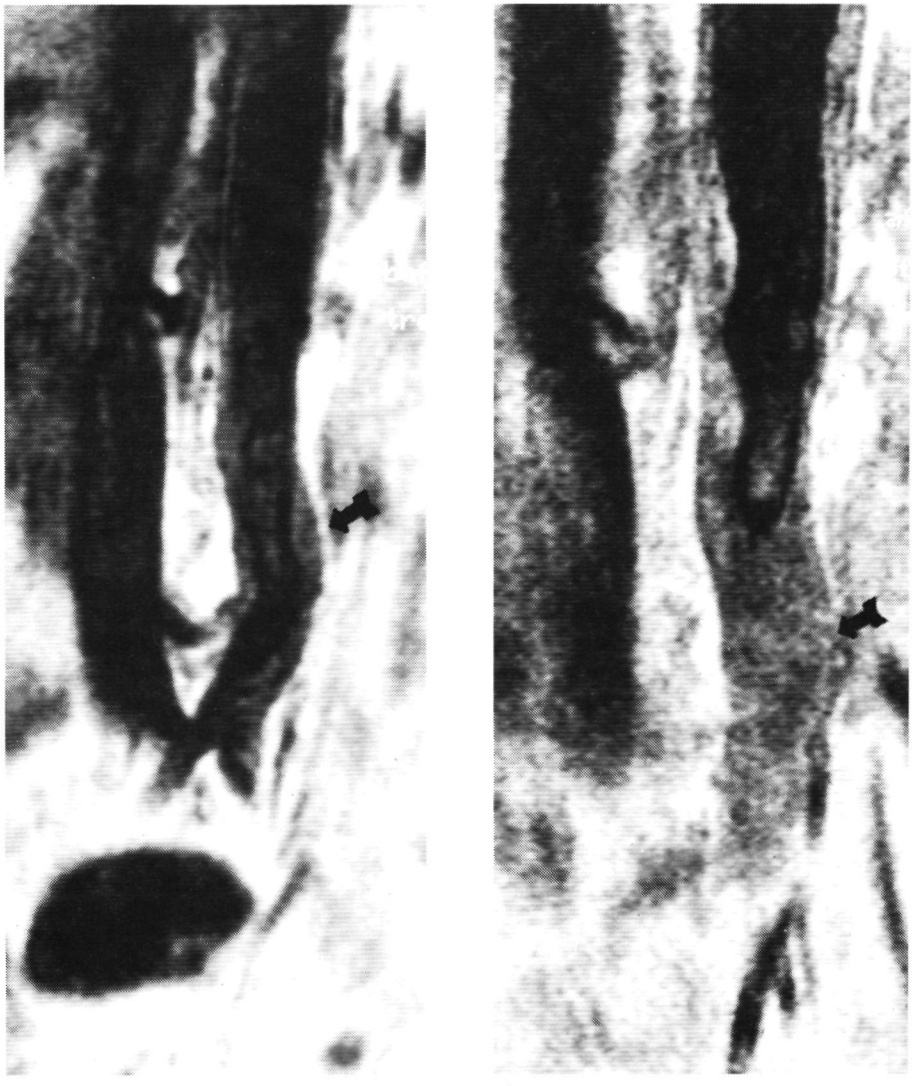
In 2 out of 6 placebo-treated patients (Table 13.1, patient number 9 and 10) the arteriosclerosis deteriorated as assessed with MRI and SBM. With SBM, the blood velocity in the right leg in patient number 9, and in both legs in patient number 10 deteriorated. The arteriosclerosis assessed with the MRI technique in patient number 9, who had only slight vascular damages at the left side of the aortic wall of 5%, was also progressive, resulting in arterioslerotic changes at the left aortic wall to 25% (Figure 13.1a and 13.1b). In patient number 10, who showed on the baseline MRI large arteriosclerotic plaques upto 60% in the abdominal aorta and left common iliac artery (Figure 13.2a and 13.2b), the vascular damage was that much deteriorated that the abdominal aorta was occluded completely after two years of placebo treatment (Figure 13.1b). The placebo-treated patient number 11 with CVD on the basis of right common carotid artery stenosis of 50% to 75%, showed deterioration after two years to complete occlusion of this arterial segment with retrograde blood flow in the right external carotid artery In that same patient, the SBM revealed regression of the arteriosclerosis at both lower extremities. The arteriosclerosis in this specific patient was defined as progressive. In the placebo-treated patient number 12 the arteriosclerosis improved by means of SBM in rest at the right side, and after occlusion in both legs. In the resulting 2 placebo-treated patient (Table 13 1; patient number 13 and 14) no vascular changes were determined.



a

b

Figure 13.1a and 13.1b. A 40 year old patient showed no arteriosclerotic damages on the baseline MRI (Figure 13.1a). After two years of placebo-treatment (Figure 13.1b), an arteriosclerotic plaque was visual at the left side of the abdominal aorta.



a b

Figure 13.2a and 13.2b. A female 46 year old patient showed severe arteriosclerotic damages on the baseline MRI (Figure 13.2a) with arteriosclerotic plaques to 60% in the abdominal aorta and left common iliac artery. After two years of placebo-treatment (Figure 13.2b), the vascular damages were such deteriorated that the abdominal aorta was occluded completely.

Discussion

Mild hyperhomocysteinemia is an established risk factor for premature arteriosclerosis In this study, more than half of hyperhomocysteinemic vascular patients revealed vascular changes at segments from which no clinical symptoms originated Therefore, arteriosclerosis in these relatively young patients is likely to be generalized[23].

Blood homocysteine, vitamin B_6 and folic acid concentrations were measured as an indication of the compliance of the patients in taking the prescribed vitamin treatment The homocysteine, vitamin B_6 and folic acid levels after one and two years were about equal as the baseline levels in the placebo-treated patients, showing that they did not use vitamin supplementation prescribed by another doctor or bought in a drug store.

Vitamin B_6, 250 mg daily, as a sole therapy normalizes the exaggerated rise after methionine loading of homocysteine only in about 56%[12], but does not affect fasting hyperhomocysteinemia[24] Nowadays, it is shown that a lower dose of vitamin B_6 of 100 mg daily has the same effect[25]. Folic acid treatment solely in a dose as low as 0 65 mg daily, can reduce elevated fasting homocysteine levels with about 40%[26] A dose of 5 mg daily may not lead to a substantially higher decrease[25 27]. However, at the time of the start of this study, folic acid was only available in tablets of 5 mg Therefore, we have supplemented the studied patients with 250 mg vitamin B_6 and 5 mg daily folic acid daily According to reports in literature, this combined supplementation will normalize elevated fasting and post-load homocysteine levels in more than 90% of patients[13] The mean reductions obtained in the present study by this combination of supplementation of fasting and post-load homocysteine, 58% and 51%, respectively, were comparable with earlier findings by van den Berg et al.[13], who administered the same dose of this combination, and by Dudman et al [27], after supplementation of only 100 mg of vitamin B_6 and 5 mg of folic acid daily

The extensive experience with biochemical effects of such vitamin treatment in mild hyperhomocysteinemia contrasts very much with lack of knowledge of clinical significance of such treatment in terms of preventing the arteriosclerotic progress. Preliminary data of the present double-blind, placebo-controlled intervention study should be enterpreted with caution because of the still small number of included patients. Three out of 6 placebo-treated patients showed progression of arteriosclerosis, whereas the arteriosclerosis in 2 other placebo-treated patients remained unchanged and showed regression only in one In case of combined vitamin B_6 and folic acid supplementation had no effect at all, the same percentage of number of patients with progression of arteriosclerosis as observed in the placebo-treated patients, would have been expected in the vitamin-treated group of patients, i.e 4 out of the 8 patients. However, progression in the vitamin-treated group of patients was established in not more than one out of eight vitamin-treated patients (12.5%) Although the number of patients in this study is quite small, significance of the difference between these effects could be calculated ($p < 0\ 05$, chi-square) These findings suggest that combined vitamin B_6 and folic acid treatment might be capable to prevent progression of arteriosclerotic disease in most vascular patients with mHH

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References

1. Boushey CJ, Bereford SAA, Omenn GS, Motulsky AG. A quantitative assessment of plasma homocysteine as a risk factor for vascular disease. Probable benefits of increasing folic acid intakes. JAMA 1995,274:1049-1057.
2. Stampfer MJ, Malinow MR, Willett WC, Newcomer LM, Upson B, Ullman D. A prospective study of plasma homocyst(e)ine and risk of myocardial infarction in US physicians. JAMA. 1992;268:877-881.
3. Arnesen E, Refsum H, Bonaa KH, Ueland PM, Forde OH, Nordrehaug JE. Serum total homocysteine and coronary heart disease. Int J of Epidem 1995,24:704-709.
4 Perry IJ, Refsum H, Ebrahim SB, Ueland PM, Shaper AG. Prospective study of serum total homocysteine concentration and risk of stroke in middle-aged British men. Lancet 1995;346:1395-1398.
5. Boers GHJ, Smals AGH, Trijbels JMF, Fowler B, Bakkeren JAJM, Schoonderwaldt HC, Kleijer WJ, Kloppenborg PWC. Heterozygosity for homocystinuria in premature peripheral and cerebral occlusive arterial disease N Engl J Med 1985;313:709-715.
6. Clarke R, Daly L, Robinson K, Naughten E, Cahalane S, Fowler B, Graham I. Hyperhomocysteinemia: an independent risk factor for vascular disease. N Engl J Med 1991;324·1149-1155.
7. Kang SS, Wong PWK, Susmano A, Sora J, Norusis M, Ruggie N. Thermolabile methylenetetrahydrofolate reductase. an inherited risk factor for coronary artery disease. Am J Hum Genet 1991;48:536-545.
8. Kang S-S, Passen EL, Ruggie N, Wong PWK, Sora H. Thermolabile defect of methylenetetrahydrofolate reductase in coronary artery disease. Circ 1993,88:1463-1469.
9 Engbersen AMT, Blom HJ, Franken DG, Boers GHJ, Stevens EMB, Poirot CM, Trijbels JMF. Thermolabile 5,10-methylenetetrahydrofolate reductase as a cause of mild hyperhomocysteinemia. Am J Hum Genet 1995;56:142-150.
10. Frosst P, Blom HJ, Milos R, Goyette P, Sheppard CA, Matthews RG, Boers GHJ, den Heijer M, Kluijtmans LAJ, van den Heuvel LP, Rozen R. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. Nat Genet 1995;10:111-113.
11. Kluijtmans LAJ, van den Heuvel LPWJ, Boers GHJ, Frosst P, Stevens EMB, van Oost BA, den Heijer M, Trijbels JMF, Rozen R, Blom HJ. Molecular genetic analysis in mild hyperhomocysteinemia: a common mutation in the methylenetetrahydrofolate reductase gene is a genetic risk factor for cardiovascular disease. Am J Hum Genet 1996;58.35-41.
12. Franken DG, Boers GHJ, Blom HJ, Trijbels JMF, Kloppenborg PWC Treatment of mild hyperhomocysteinemia in vascular disease patients. Arterioscler Thromb 1994;14;465-470.
13. van den Berg M, Franken DG, Boers GHJ, Blom HJ, Jacobs C, and Rauwerda JA. Combined vitamin B_6 and folic acid therapy in patients with premature arteiosclerosis and mild hyperhomocysteinemia. J Vasc Surg 1994;20:933-940.
14. van den Berg M, Boers GHJ, Franken DG, Blom HJ, van Kamp GJ, Jacobs C, Rauwerda JA, Kluft C and Stehouwert CDA. Hyperhomocysteinemia and endothelial dysfunction in young patients with peripheral arterial occlusive disease Eur J Clin Invest 1995;25:176-181.
15. Mudd SH, Levy HL, Skovby F. Disorders of transsulfuration. In Scriver CR, Beaudet AL, Sly WS, Valle D (eds). The metabolic basis of inherited disease McGraw-Hill Inc., New York, 1995;1279-1327.
16. Boers GHJ, Fowler B, Smals AGH, Trijbels JMF, Leermakers AI, Kleijer WJ, Kloppenborg PWC. Improved identification of heterozygotes for homocystinuria due to cystathionine synthase deficiency by the combination

of methionine loading and enzyme determination in cultured fibroblasts. Hum Genet 1985;69:164-169.
17. te Poele-Pothoff MTWB, van den Berg M, Franken DG, Boers GHJ, Jakobs C, de Kroon IFI, Eskes TKAB, Trijbels Three different methods for the determination of total homocysteine in plasma. JMF, Blom HJ. Annals of Clin Biochem 1995;32:218-220.
18. Boers GHJ, Smals AGH, Trijbels JMF, Leermakers AI, Kloppenborg PWC. Unique efficiency of methionine metabolism in premenopausal women may protect against vascular disease in the reproductive years. J Clin Invest 1983;72:1971-1976.
19. Wouters MGAJ, Moorrees MThEC, Van der Mooren MJ, Blom HJ, Boers GHJ, Schellekens LA, Thomas CMG, Eskes TKAB. PLasma homocysteine and menopausal status. Eur J CLin Invest 1995;25:801-805.
20. Kempczinski RF, Yao JST. In: Practical noninvasive vascular diagnosis. Year Book Medical Publishers, Inc., Chigaco, 1987.
21. Strandness DE. Duplex scanning in vascular disorders. Raven Press Inc., New York, 1993, 2nd ed.
22. Franken DG, Barentsz JO, Heystraten FMJ, Boers GHJ, and Ruijs JHJ. Imaging of Vascular Pathology in Hyperhomocysteinemic Patients with Digital Subtraction Angiography and Magnetic Resonance Techniques. Submitted 1996.
23. van den Berg, Stehouwer CDA, Bierdrager E, Rauwerda JA. Plasma homocysteine and severity of atherosclerosis in young patients with lower extremity atherosclerotic disease. Arterioscler Thromb Vasc Biol 1996;16:165-171.
24. Brattstrom L, Israelsson B, Norrving B, Bergqvist D, Thorne J, Hultberg B, Hamfelt A. Impaired homocysteine metabolism in early-onset cerebral and peripheral occlusive arterial disease. Atherosclerosis 1990,81:51-60.
25. Boers GHJ, van den Berg M, Franken DG. Treatment of mild hyperhomocysteinemia. In: Homocysteine metabolism, from basic science to clinical medicine. Kluwer Academic Publishers, 1996; In Press.
26. Ubbink JB, Vermaak WJH, van der Merwe A, Becker PJ, Delport R, Potgieter HC. Vitamin requirements for the treatment of hyperhomocysteinemia in humans. J Nutr 1994;124:1927-1933.
27. Dudman NPB, Wilcken DEL, Wang J, Lynch JF, Macey D, Lundberg P. Disordered methionine/homocysteine metabolism in premature vascular disease. Its occurrence, cofactor therapy, and enzymology. Arterioscler Thromb 1993;13:1253-1260.

General discussion and future perspectives

Mild hyperhomocysteinemia has been established in vascular patients in numerous studies with a frequency varying from 9% to 47%, as reviewed extensively by Ueland et al.[1] and Kang et al.[2]. From pooled data, it can be estimated that one in four to five patients with symptomatic arteriosclerosis will show mild hyperhomocysteinemia[3,4]. This prevalence corresponds well with our percentages of mild hyperhomocysteinemia among patients with coronary, cerebrovascular, and peripheral vascular disease, as described in Chapters 8 and 9 of this thesis[5,6] Comparably mild elevations of homocysteine blood levels can be measured in about 5% to 8% of healthy subjects[1,2]. So, mild hyperhomocysteinemia is a common risk factor for cardiovascular disease

The findings from the investigations described in this thesis are discussed in the light of recently reported relevant studies and are put into future perspectives. The following questions will be dealt with:

- are fasting or post-load homocysteine levels or both required to diagnose mild hyperhomocysteinemia?
- to what extent is mild hyperhomocysteinemia genetically based?
- by what mechanism(s) is mild hyperhomocysteinemia causing vascular disease?
- how to treat mild hyperhomocysteinemia?
- will homocysteine-lowering intervention be clinically efficacious?

Are fasting or post-load homocysteine levels or both required to diagnose mild hyperhomocysteinemia?

Quantitative meta-analysis of 27 studies published before 1995 relating homocysteine to arteriosclerotic vascular disease, showed a combined odds ratio as an estimate of the relative risk of elevated fasting homocysteine blood levels for coronary artery disease of 1.8 (95% CI 1.6 - 2 0), for cerebrovascular disease of 2.3 (95% CI 1 8 - 2 9), and for peripheral vascular disease of 6 5 (95% CI 2 9 - 15 8)[12] The odds ratios of all included elevated homocysteine levels, either fasting or basal or post-load, did not differ from these of the fasting elevated levels, suggesting an equally strong relative risk of fasting and post-load hyperhomocysteinemia. Furthermore, from a large multi-centre study including 750 vascular patients and 800 controls, it could also be calculated that the odds ratio for vascular disease of increased homocysteine blood levels in the fasting state is about equal to the odds ratio of increased post-load levels, i.e 2.2 versus 2 1[13]. By combining fasting ánd post-load hyperhomocysteinemia the relative risk of vascular disease increased to 3[13].

From studies in which methionine loading was used to define mild hyperhomocysteinemia, it can be calculated in what frequency post-load hyperhomocysteinemia was accompanied by elevated fasting levels Such concomitance was found varying from 37% to 59%[7,11]. This finding is in concordance with the frequency of 46% of the combination of fasting and post-load hyperhomocysteinemia as found in our study described in Chapter 9[6]. Therefore, if only the fasting homocysteine level is determined in the individual vascular patient, then a considerable number of patients with exclusively post-load hyperhomocysteinemia will be missed. Therefore, in our opinion, it is not justifiable to abolish the standardized methionine loading test as a determinant of mild

hyperhomocysteinemia in individual vascular patients. The performance of a methionine loading test in epidemiological studies in large groups of patients may be too laborious, and the determination of fasting or even non-fasting "basal" level can be an alternative.

Possible simplifications of the methionine loading test should be explored. Shortening the methionine loading test by determining the plasma homocysteine concentration 2 or 4 hours after methionine intake might be an option[14]. Another possibility to simplify the screening could also be to skip sampling of fasting blood for homocysteine determination and to draw blood 6 hours after the patient has taken methionine at home. The patient may use a light breakfast, because 3 studies showed that such meal will give no or only a minor change in the homocysteine blood levels[15-17]. Skipping sampling of fasting blood, this screening would fail to detect no more than about 6 vascular patients with fasting hyperhomocysteinemia among 100 with post-load normohomocysteinemia (own observation, n = 269). This is in line with the conclusion by Bostom et al.[10] who found increased fasting blood homocysteine levels in 17 out of 227 (7.5%) subjects with normal plasma homocysteine concentration 4 hours after oral methionine load. Fasting homocysteine level was elevated in only 2 out of 60 (3.3%) post-load normohomocysteinemic family members of post-load hyperhomocysteinemic vascular patients as described in Chapter 4[18], which low percentage could, however, been biased because only family members of post-load hyperhomocysteinemic patients were included in that study, regardless their fasting homocysteine concentration.

Individuals with pathologically high homocysteine blood levels in the fasting state and normal post-load levels may represent a subgroup different from those with both fasting and post-load hyperhomocysteinemia, or different from that with post-load hyperhomo-cysteinemia and fasting normohomocysteinemia. Such a diversity which can only be disclosed by a methionine loading test, may be due to heterogeneity in enzymatic defects underlying hyperhomocysteinemia. In case future studies will confirm such diversity, it may also be warranted in these subgroups to prescribe specific homocysteine-lowering regimen.

To what extent is mild hyperhomocysteinemia genetically based?

The conclusion from data as presented in Chapters 4, 5 and 6 of this thesis[18-20] is in line with previous reports[21-25] that mild hyperhomocysteinemia is at least partially genetically based. Low blood vitamin B_6, vitamin B_{12} and folic acid concentrations, genetically or environmentally determined, can also lead to elevated plasma homocysteine concentration[1,26,27]. Non-genetic causes, such as liver and renal diseases can also increase blood homocysteine levels[1]. In a study which is presented in Chapter 4 among family members of 21 post-load hyperhomocysteinemic vascular patients, after excluding vitamins deficiencies and liver and renal diseases in the index patients, it is shown that the chance to find pathologically high homocysteine levels is much higher in the family members than might be expected in the normal population[18]. Post-load mild hyperhomocysteinemia was established in at least one other family member in 71% of the families, indicating a strong genetic base of the mild hyperhomocysteinemia. Thermolability of MTHFR, associated with decreased enzyme activity[20] and based upon homozygosity for 677 C→T mutation in the gene for this enzyme on

chromosome 1[28], could be established in 28% of vascular patients with mild hyperhomocysteinemia, as presented in Chapter 5[20]. Cystathionine ß-synthase deficiency has been shown to be present in none or only a very small minority of vascular patients with mild hyperhomocysteinemia[20,29,30]. Therefore, other genetic defects must account for the majority of mild hyperhomocysteinemia as detected among vascular patients. Deficiency of activity of homocysteine remethylation enzymes, i.e. methionine synthase and betaine-homocysteine methyltransferase, and inborn errors in folic acid and vitamin B_{12} metabolism, especially in the synthesis of their substrates and cofactors relevant for methionine-homocysteine metabolism, are to be focused upon as potential genetical origins of hyperhomocysteinemia.

By what mechanism(s) is mild hyperhomocysteinemia causing vascular disease?

Although the sulphydryl containing amino acid homocysteine is generally held to be an atherogenic and thrombotic agent, there is no consensus about the pathophysiological mechanism, as has been discussed in Chapter 2. Some hypotheses based on results from in vitro studies are promising, but the extremely high homocysteine levels at which these investigations have been performed, are not reflecting the physiological state in mild or even severe hyperhomocysteinemia in man. Apart from this, not one hypothesis is unifying clearly the atherogenic and thrombophilic tendency of hyperhomocysteinemia. The well known cases of classical homocystinuria with severely elevated blood homocysteine levels, despite homocysteine-lowering treatment, who remain free of arteriosclerotic or thromboembolic events during several decades of follow-up are in this respect very enigmatic[31]. Possible synergistic interactions of elevated blood homocysteine concentration with other risk factors for vascular disease are very interesting. Such synergism, leading to expression of vascular disease, occurred in those family members with concomitant protein C deficiency ánd mild hyperhomocysteinemia in a family with both conditions inherited, as described by us in Chapter 6[19]. In line with this observation is the recently reported synergism between severe hyperhomocysteinemia and factor V Leiden mutation leading to a resistance against activated protein C[32]. In this study, only those homocystinuric patients suffered from arterial and venous thrombosis or pulmonary embolismin case factor V Leiden mutation was also present.

How to treat mild hyperhomocysteinemia?

After reviewing available studies until now on homocysteine-lowering treatment as described in Chapter 7[33], the use of folic acid, 0.65 mg daily, seems to be capable to decrease elevated fasting blood levels of homocysteine with about 40%, and the combined use of 100 mg vitamin B6 and 5 mg folic acid daily can lower the post-load homocysteine levels with about 50%. Normalization of elevated post-load homocysteine levels in blood is obtained by daily 250 mg vitamin B_6 solely in about half of the vascular patients as described in Chapter 8[5]. Combined vitamin B_6, 250 mg daily, and folic acid, 5 mg daily, surpasses this effect because it leads to a normalization of the fasting and the post-load increased homocysteine levels in almost all vascular hyperhomocysteinemic patients, as reported in Chapters 9 and 11[6,7]. Future dose-response studies should clarify the

lowest required dosage. Clinical adverse effects of these two vitamins in the dosages as presently used, are not reported[34-38]. Therefore, dose-response studies do not have the highest precedence.

Summarizing, fasting and post-load hyperhomocysteinemia as present in vascular patients can be treated safely and easily with folic acid and vitamin B_6 at the aforementioned dosages. In rare non-responsive hyperhomocysteinemic patients, there is need for other options for therapy. One option could be the prescription of betaine, which has been discussed in Chapter 7[33]. This substance, however, is a non-registered drug and can not be prescribed in most countries. In the study described in Chapter 11[39], the use of vitamin B_1 therapy as alternative homocysteine-lowering intervention is explored. The active form of thiamine, thiamine pyrophosphate, is a cofactor of the supposed rate-limiting oxidative decarboxylation in the transamination pathway of methionine. A dose up to 75 mg of vitamin B_1 daily showed no significant reduction of fasting homocysteine concentration in homocystinuric patients who had still high homocysteine blood levels despite conventional therapy. This finding does not make a beneficial effect of such treatment in vascular patients with mild hyperhomocysteinemia likely.

Will homocysteine-lowering intervention be clinically efficacious?

Screening for mild hyperhomocysteinemia in vascular patients and prescribing homocysteine-lowering treatment at a large scale, will only be justified if a clinically beneficial effect of homocysteine-lowering treatment has been established. In Chapter 11[7] we describe that the combined use of vitamin B_6 and folic acid as homocysteine-lowering treatment during one year is capable to significantly decrease elevated von Willebrand factor and thrombomodulin, both markers of endothelial dysfunction, which are elevated in hyperhomocysteinemic vascular patients. This result is suggestive of a redression of endothelial damage by such intervention. However, this study was not placebo controlled, so the presence of elevated endothelial markers on the base of the vascular event itself can not be ruled out. For the future, randomized clinical trials, to prove a beneficial effect of homocysteine-lowering treatment, should be given the highest priority. Studies to explore the reduction of number of recurrent clinical events in treated hyperhomocysteinemic vascular patients will require a large number of included patients and a long follow-up period. Therefore, the development of methods to measure minimal changes of arteriosclerotic lesions should be continued and the accuracy of these techniques should be thoroughly evaluated. In this regard, as described in Chapter 12, the accuracy of Magnetic Resonance Imaging (MRI) and Magnetic Resonance Angiography (MRA) techniques were compared with intra-arterial digital subtraction angiography (iaDSA) procedures in 11 mildly hyperhomocysteinemic patients with an established vascular event at young age. It could be concluded that MRI in such patients appears to be an acceptable imaging technique compared with iaDSA, and may be an alternative method to the invasive iaDSA technique. MRA, however, does not seem to provide significant additional information to MRI. Preliminary results of our placebo-controlled double-blind prospective intervention study with the use of vitamin B_6 and folic acid as homocysteine-lowering agent in 14 hyperhomocysteinemic vascular patients are presented in Chapter 13. It is concluded that such intervention may have a potency to prevent progression of arteriosclerosis. The outcome of ongoing studies is

mandatory to prove such clinically effect definitively.

Assuming that homocysteine-lowering treatment is effective in reducing the progression of arteriosclerosis in vascular hyperhomocysteinemic patients, then it will be likely that such intervention prevents arteriosclerosis in the general population. If so, it can be calculated for the general population in the United States of America, that if folic acid intake is increased by fortification of flour and cereal products by using 350 μg folic acid per 100 g of such food daily, 9% coronary heart deaths in men and 5% in women could potentially be prevented[12,40]. In 1993 in the Netherlands, 12,691 men and 9,427 women have died from acute myocardial infarction[41]. So, under the proviso that the estimate for the USA is valid in the Netherlands as well, about 1,600 patients could be saved from coronary death yearly. Speculating that the same percentage of death prevention is applicable in cerebrovascular and peripheral arterial disease, about 1000 more deaths could be prevented yearly.

References

1. Ueland PM, Refsum H, Brattström L. Plasma homocysteine and cardiovascular disease. In: Francis RBF (ed). Atherosclerotic cardiovascular disease, hemostasis, and endothelial function. Marcel Dekker Inc., New York, 1992;183-236.
2. Kang S-S, Wong PWK, Malinow MR. Hyperhomocysteinemia as a risk factor for occlusive vascular disease. Annu Rev Nutr 1992;12:279-298.
3. Ueland PM, Refsum H, Stabler SP, Malinow MR, Andersson A, Allen RH. Total homocysteine in plasma or serum: Methods and clinical applications. Clin Chem 1993;39:1764-1779.
4. Boers GHJ. Hyperhomocysteinaemia: a newly recognized risk factor for vascular disease. Neth J Med 1994;45:34-41.
5. Franken DG, Boers GHJ, Blom HJ, Trijbels JMF, Kloppenborg PWC. Treatment of mild hyperhomocysteinemia in vascular disease patients. Arterioscl Thromb 1994;14:465-470.
6. van den Berg M, Franken DG, Boers GHJ, Blom HJ, Jacobs C, Rauwerda JA. Combined vitamin B_6 and folic acid therapy in patients with premature arteriosclerosis and mild hyperhomocysteinemia. J Vasc Surg 1994;20:933-40.
7. van den Berg M, Boers GHJ, Franken DG, Blom HJ, van Kamp GJ, Jacobs C, Rauwerda JA, Kluft C and Stehouwert CDA. Hyperhomocysteinemia and endothelial dysfunction in young patients with peripheral arterial occlusive disease. Eur J Clin Invest 1995;25:176-181.
8. Fermo I, D'Angelo SV, Paroni R, Mazzola G, Calori G, D'Angelo A. Prevalence of moderate hyperhomocysteinemia in patients with early-onset venous and arterial occlusive disease. Ann Intern Med 1995;123:747-753.
9. den Heyer M, Blom HJ, Gerrits WBJ, Rosendaal FR, Haak HL, Wijermans PW, Bos GMJ. Is hyperhomocysteinemia a risk factor for recurrent venous thrombosis? Lancet 1995;345:882-885.
10. Bostom AG, Jacques PF, Nadeau MR, Williams RR, Ellison RC, Selhub J. Post-methionine load hyperhomocysteinemia in persons with normal fasting total plasma homocysteine: initial results from The NHLBI Family Heart Study. Atheroscler 1995;116:147-151.
11. Verhoef P, Hennekens CH, Allen RH, Stabler SP, Willett WC, Stampfer MJ. Plasma total homocysteine and future risk of angina pectoris with evidence of severe coronary atherosclerosis. Submitted 1996.
12. Boushey CJ, Bereford SAA, Omenn GS, Motulsky AG. A quantitative assessment of plasma homocysteine as a risk factor for vascular disease. Probable benefits of increasing folic acid intakes. JAMA 1995;274:1049-1057.
13. Daly L, Meleady R, Graham I. Fasting or post-methionine load homocysteine: which should be measured in relation to vascular risk? Irish J Med Science 1995;164(S15):6 (Abstract).
14. Bostom AG, Roubenoff R, Dellaripa P, Nadeau MR, Sutherland P, Wilson PWF, Jacques PF, Selhub J, Rosenberg IH. Validation of abbreviated oral methionine-loading test. Clin Chem 1995;41:948-949.
15. Malinow MR, Kang SS, Taylor LM, Wong PWK, Coull B, Inahara T, Mukerjee D, Sexton G, Upson B. Prevalence of hyperhomocyst(e)inemia in patients with peripheral arterial occlusive disease. Circ 1989;79:1180-1188.
16. Guttormsen AB, Schneede J, Fiskerstrand T, Ueland PM, Refsum HM. Plasma concentrations of homocysteine and other aminothiol compounds are related to food intake in healthy human subjects. J Nutr 1994;124:1934-1941.
17. Ubbink JB, Vermaak WJH, Van der Merwe A, Becker PJ. The effect of blood sample aging and food consumption on plasma total homocysteine levels. Clin Chim Acta 1992;207:119-128.
18. Franken DG, Boers GHJ, Blom HJ, Cruijsberg JRM, Trijbels JMF, Hamel BCJ.

Prevalence of familial mild hyperhomocysteinemia. Atheroscler 1996;125:71-80.
19. Franken DG, Vreugdenhil A, Boers GHJ, Verrips A, Blom HJ, Novakova IRO. Familial cerebrovascular accidents due to hyperhomocysteinemia and protein C deficiency type 1. Stroke 1993;24:1599-1600.
20. Engbersen AMT, Blom HJ, Franken DG, Boers GHJ, Stevens EMB, Poirot CM, Trijbels JMF. Thermolabile 5,10-methylenetetrahydrofolate reductase as a cause of mild hyperhomocysteinemia. Am J Hum Genet 1995;56:142-150.
21. Reed T, Malinow MR, Christian JC, Upson B. Estimates of heritability of plasma homocyst(e)ine levels in aging adult male twins. Clin Genet 1991;39:425-428.
22. Berg K, Malinow MR, Kierulf P, Upson B. Population variation and genetics of plasma homocyst(e)ine level. Clin Genet 1992;41:315-321.
23. Genest JJ, McNamara JR, Upson B, Salem DN, Ordovas JM, Schaefer EJ, Malinow MR. Prevalence of familial hyperhomocyst(e)inemia in men with premature coronary artery disease. Arterioscler Thromb 1991;11:1129-1136.
24. Williams RR, Malinow MR, Hunt SC, Upson B, Wu LL, Hopkins PN, Stults BN, Kuida H. Hyperhomocyst(e)inemia in Utah siblings with early coronary disease. Coron Art Dis 1990;1:681-685.
25. Wu LL, Wu J, Hunt SC, James BC, Vincent GM, Williams RR, and Hopkins PN. Plasma homocyst(e)ine as a risk factor for early familial coronary artery disease. Clin Chem 1994;40:552-561.
26. Brattström L, Israelsson B, Norrving B, Bergqvist D, Thörne J, Hultberg B, Hamfelt A. Impaired homocysteine metabolism in early-onset cerebral and peripheral occlusive arterial disease. Effects of pyridoxine and folic acid treatment. Atheroscl 1990;81:51-60.
27. Selhub J, Jacques PF, Wilson PWF, Rush D, Rosenberg IH. Vitamin status and intake as primary determinants of homocysteinemia in an eldery population. JAMA 1993;270:2693-2698.
28. Frosst P, Blom HJ, Milos R, Goyette P, Sheppard CA, Matthews RG, Boers GHJ, den Heijer M, Kluijtmans LAJ, van den Heuvel LP, Rozen R. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. Nat Genet 1995;10:111-113.
29. Kozich V, Kraus E, de Franchis R, Fowler B, Boers GHJ, Graham I, Kraus JP. Hyperhomocysteinemia in premature arterial disease: examination of cystathionine ß-synthase alleles at the molecular level. Hum Mol Genet 1995;4:623-629.
30. Kluijtmans LAJ, van den Heuvel LPWJ, Boers GHJ, Frosst P, Stevens EMB, van Oost BA, den Heijer M, Trijbels JMF, Rozen R, Blom HJ. Molecular genetic analysis in mild hyperhomocysteinemia: a common mutation in the methylenetetrahydrofolate reductase gene is a genetic risk factor for cardiovascular disease. Am J Hum Genet 1996;58:35-41.
31. Boers GHJ. Personal communication.
32. Mandel H, Brenner B, Berant M, Rosenberg N, Lanir N, Jakobs C, Fowler B, Seligsohn U. Coexistence of hereditary homocystinuria and factor V Leiden - effect on thrombosis. N Eng J Med 1996;334:763-768.
33. Boers GHJ, van den Berg M, Franken DG. Treatment of mild hyperhomocysteinemia. In: Homocysteine metabolism, from basic science to clinical medicine. Kluwer Academic Publishers, 1996; In press.
34. Schaumberg H, Kaplan J, Windebanke A, Vick N, Rasmus S, Pleasure D, Brown MJ. Sensory neuropathy from pyridoxine abuse. N Engl J Med 1983;309:445-448.
35. Brody T, Shane B, Stokstad ELR. Folic acid. In: Machlin LJ (ed). Handbook of vitamins, nutritional, biochemical, and clinical aspects. Marcel Dekker Inc., New York and Basel, 1984;459-496.

36. Parry GJ, Bredensen DE. Sensory neuropathy with low dose pyridoxine. Neurology 1985;35:1466-1468.
37. Friedrich W. Folic acid and unconjugated pteridines. In: Friedrich W (ed). Vitamins. Walter de Gruyter Inc., New York, 1988;619-752.
38. Butterworth CE, Tamura T. Folic acid safety and toxicity: a brief review. Am J Clin Nutr 1989;50:353-358.
39. Franken DG, Blom HJ, Boers GHJ, Tangerman A, Thomas CMG, Trijbels JMF. Thiamine (vitamin B_1) supplementation does not reduces fasting blood homocysteine concentration in homozygotes for homocystinuria. Biochim Biophys Acta 1996; In press.
40. Motulsky AG. Nutritional ecogenetics: homocysteine-related arteriosclerotic vascular disease, neural tube defects, and folic acid. Am J Hum Genet 1996;58:17-20.
41. Dutch Heart Foundation. Heart and vascular diseases in the Netherlands. Morbidity and mortality statistics. The Hague: Dutch Heart Foundation, (Dutch) 1995.

Summary

In general health care vascular disease is a major disorder with a high prevalence, resulting in considerable morbidity and medical costs. During the last two decades, it became overwhelmingly clear that mild hyperhomocysteinemia has to be recognized as one of the risk factors for arteriosclerotic and thrombotic diseases.

The aims of the studies presented in this thesis were to investigate whether mild hyperhomocysteinemia in vascular patients is genetically determined, i.e which genetic defects are the basis for this risk factor for cardiovascular disease, and what is their prevalence (Part 1). Furthermore, it was explored if elevated blood homocysteine concentrations can be lowered or even normalized by the use of B-vitamins in vascular patients with mild hyperhomocysteinemia (Part 2), and whether this treatment may result in a clinically beneficial outcome (Part 3).

After a general introduction and the presentation of the objectives of this thesis (**Chapter 1**) an update on hyperhomocysteinemia is presented (**Chapter 2**), and laboratory assays of homocysteine in plasma have been described (**Chapter 3**). All 3 investigated methods for measuring the concentration of homocysteine in blood, i.e. one by means of a conventional amino acid analyzer and two by using high-performance liquid chromatography (HPLC), are appropriate. HPLC sodium borohydride/monobromobimane technique may be preferable because this method is fully automated and has a large sample capacity.

In part 1 in **Chapter 4**, the prevalence of mild hyperhomocysteinemia in families of which one family member was known with vascular disease and mild hyperhomocysteinemia was studied. We measured fasting and post-load homocysteine levels among 96 family members of 21 post-load hyperhomocysteinemic vascular index patients. In 71% of the 21 screened families, post-load elevated homocysteine level could be established in at least one other family member. Fasting and post-load mild hyperhomocysteinemia in these family members was observed in 21% and 32%, respectively. It was concluded that both fasting and post-load mHH seems to be inherited in the majority of hyperhomocysteinemic vascular patients.

In **Chapter 5**, the method of determination and prevalence of thermolabile mutant variant of methylenetetrahydrofolate reductase (MTHFR) among vascular patients with mild hyperhomocysteinemia has been reported. In 28% of such patients their mild hyperhomocysteinemia was caused by thermolabile mutant variant of MTHFR, which is determined by a decrease of enzyme activity and increased inactivation after incubation at 46 °C.

The occurrence of two inherited risk factors for vascular disease, i e. mild hyperhomocysteinemia and protein C deficiency type 1, within one family has been described in **Chapter 6**. Only those family members exhibiting the two risk factors combined revealed symptomatic vascular disease. From this family a synergistic interaction between these atherogenic and thrombogenic risk factors is suggested.

The biochemical effect of homocysteine-lowering treatment is explored in Part 2, Chapters 7 to 9.

Firstly, a review of the literature on homocysteine-lowering regimen until 1996 is reported in **Chapter 7**. Elevated fasting homocysteine concentration in blood can be reduced with 40% to 50% by folic acid supplementation, in a dose of 0.65 mg to 5 mg daily, respectively. A higher dosage of folic acid up to 10 mg daily has no further effect on the decline of elevated fasting homocysteine concentration. Vitamin B_6 supplementation has no effect on fasting homocysteine levels. For the reduction of post-load hyperhomocysteinemia, the use of solely vitamin B_6, in a dose of 100 mg to 250 mg, showed a reduction of 40%. Combined treatment of vitamin B_6, 100 mg daily, and folic acid, 5 mg daily, surpasses this effect and can lead to 50% reduction. Adding orally daily 0.4 mg vitamin B_{12} may avoid deterioration of unrecognized vitamin B12 deficiency (perniciosa) after starting folic acid treatment.

From data presented in **Chapter 8**, the prevalence of post-load mild hyperhomocysteinemia in vascular patients, i.e. cerebral, peripheral or coronary artery occlusive disease, and the effect of vitamin B_6 as homocysteine-lowering treatment are described and the figures were in concordance with data from literature so far. In the period of January 1980 until December 1990, 421 patients under 55 years of age with documented premature peripheral or cerebral occlusive arterial disease had been screened for mild hyperhomocysteinemia by oral methionine loading tests at the University Hospital Nijmegen. Thirty-three percent of these patients with peripheral, and 20% of these patients with cerebral occlusive arterial disease were identified with mild hyperhomocysteinemia. Post-load hyperhomocysteinemic patients were treated with vitamin B_6, 250 mg daily for 6 weeks, which resulted in a normalization of the post-load hyperhomocysteinemia in 56% of the treated patients.

In **Chapter 9**, 309 patients with peripheral, cerebral, or coronary artery occlusive disease, diagnosed at the University Hospital of the Free University of Amsterdam, were included to establish the prevalence of mild hyperhomocysteinemia, fasting as well as in the post-load state. Mild post-load hyperhomocysteinemia was detected overall in 23% of the patients, of whom about 50% also had fasting hyperhomocysteinemia. Increased fasting and/or post-load homocysteine concentrations could be normalized in more than 90% of the hyperhomocysteinemic patients by combined vitamin B_6 and folic acid supplementation, 250 mg and 5 mg daily, respectively. It could be concluded from Chapter 8 and 9 that mild hyperhomocysteinemia is a frequently encountered risk factor for arteriosclerosis, which can easily and inexpensively be treated by vitamin B_6 and folic acid.

As presented in **Chapter 10**, vitamin B_1 supplementation to stimulate transamination of methionine has been administered to nine homozygotes for homocystinuria, with persistence of their elevated homocysteine levels in spite of conventional homocysteine-lowering treatment. In case such supplementation would normalize elevated homocysteine concentration, it might also be an effective treatment in those cases in whom the mild hyperhomocysteinemia is not

responding to treatment of vitamin B_6, folic acid or both. However, vitamin B_1 does not lower the homocysteine concentration in 8 out of the 9 studied homozygotes for homocystinuria, and therefore, is likely not an alternative treatment in patients with mild hyperhomocysteinemia.

In Part 3 of this thesis, Chapters 11 to 13, the clinical effect of homocysteine-lowering treatment is described.

The amelioration of two markers of endothelial function, i.e von Willebrand factor and thrombomodulin, achieved by one year of combined vitamin B_6 and folic acid therapy in patients with peripheral arterial occlusive disease in the presence of mild hyperhomocysteinemia is described in **Chapter 11**. Tissue-type plasminogen activator, another endothelium-derived protein, was normal at baseline and remained unchanged after one year of treatment.

In **Chapter 12**, the abdominal aorta and proximal segments of the common iliac arteries assessed by gated T1-weighted Magnetic Resonance Imaging (MRI), and gated 2D-time of flight Magnetic Resonance Angiography (MRA) were compared with the same arterial segments assessed by intra-arterial Digital Subtraction Angiography (iaDSA) in 11 patients with proven arterial occlusive disease and mild hyperhomocysteinemia. Six patients showed arteriosclerosis in one or more arterial segments by means of iaDSA. Eighty percent of the segments with MRI, and 64% of the segments with MRA were classified correctly. The sensitivity, specificity and accuracy of MRI could be calculated as 67%, 86%, and 80%, respectively. MRA could not achieve these results. It can be concluded that signs of generalized arteriosclerosis are frequently present in hyperhomocysteinemic patients, and MRI in such patients is an acceptable non-invasive technique to use as a diagnosticum for detection of even small arteriosclerotic lesions.

In a two-year running, placebo-controlled, double-blind prospective intervention study with combined vitamin B_6 and folic acid treatment in 14 vascular patients with mild hyperhomocysteinemia, the clinical effect of such homocysteine-lowering treatment was studied and presented in **Chapter 13**. As one of the non-invasive methods to measure progression or regression of arteriosclerotic changes of vessel walls after 2 years of intervention, the MRI technique as described in Chapter 12 was used. Three out of 6 placebo-treated patients and 1 out of 8 vitamin-treated patients showed progression of arteriosclerosis after two years of daily placebo or combined vitamin B_6 and folic acid treatment, respectively. Moreover, one out of 6 placebo-treated, and four out of 8 vitamin-treated patients showed regression of arteriosclerosis. Although the number of studied patients is small, statistical significance of the effect on arteriosclerosis by the homocysteine-lowering regimen compared to placebo-treatment was found. Thus, combined vitamin B_6 and folic acid treatment might even be capable to redress arteriosclerotic vessel wall changes in hyperhomocysteinemic patients.

In **Chapter 14**, it is discussed how the objectives of this thesis have been met by the presented results and future perspectives are indicated.

Samenvatting

Vaataandoeningen komen in de algemene gezondheidszorg vaak voor, resulterend in een redelijk hoge overlijdingskans van de patiënten hoge medische kosten voor de gemeenschap. De afgelopen twee decennia is het duidelijk geworden dat een milde verhoging van het homocysteïne-gehalte in het bloed, een zogenaamd "milde hyperhomocysteïnemie", één van de risicofactoren voor vaatverkalking (arteriosclerose) en thrombose (bloedstolsels) is.

Het doel van de studies, beschreven in dit proefschrift, was te onderzoeken of milde hyperhomocysteïnemie voornamelijk erfelijk bepaald is of door omgevingsfactoren veroorzaakt wordt. Welke erfelijke defecten zijn de oorzaak voor deze risicofactor voor hart- en vaatziekten en hoe vaak komen deze voor (Deel 1, hoofdstukken 4 tot 6)? Bovendien is onderzocht of een verhoogd homocysteïne-gehalte in het bloed verlaagd of zelfs tot binnen normale waarden gebracht kan worden door het gebruik van vitamine B_6, eventueel in combinatie met foliumzuur (= vitamine B_{11}) bij patiënten met vaatverkalking en milde hyperhomocysteïnemie (Deel 2, hoofdstukken 7 tot 10), en tenslotte, of deze vitamine B-therapie een klinisch gunstig effect heeft (Deel 3, hoofdstukken 11 tot 13). Anders gezegd, kan vitamine B als homocysteïne-verlagende therapie de toename van vaatverkalking doen afnemen?

Na een korte algemene introductie (**Hoofdstuk 1**) en een overzicht van wat er bekend is op het gebied van hyperhomocysteïnemie (**Hoofdstuk 2**), zijn laboratoriumtechnieken om het homocysteïne-gehalte in het bloed te meten beschreven (**Hoofdstuk 3**). De 3 onderzochte methoden blijken alle geschikt te zijn. De voorkeur gaat echter uit naar één van de beschreven technieken, aangezien deze volledig geautomatiseerd is en veel bloedmonsters per dag kan verwerken.

In **Hoofdstuk 4** is de frequentie van vóórkomen van milde hyperhomocysteïnemie bestudeerd bij families van wie één familielid met vaatverkalking en milde hyperhomocysteïnemie bekend is. We hebben de homocysteïne- concentratie zowel nuchter als na methionine belasting (een test die de methionine-homocysteïne stofwisseling belast) bepaald bij 96 familieleden van 21 patiënten met arteriosclerose en milde hyperhomocysteïnemie. Bij 71% van de 21 onderzochte families kon bij minstens één ander familielid een verhoogd homocysteïne-gehalte in het bloed vastgesteld worden. Bij deze familieleden werd milde hyperhomocysteïnemie bij 21% in nuchtere toestand en bij 32% na methionine belasting geconstateerd. We concludeerden dat milde hyperhomocysteïnemie erfelijk is bij de meeste patiënten met vaatverkalking.

In **Hoofdstuk 5** wordt de methode beschreven, waarmee een tekort in activiteit van de thermolabiele variant van methyleentetrahydrofolaat reductase kan worden vastgesteld. Dit is een enzym in het homocysteïne metabolisme, dat bij een verlaagde activiteit tot een verhoogde homocysteïne-concentratie in bloed kan leiden. Bij 28% van hyperhomocysteïnemische patiënten met arteriosclerose bleek de milde hyperhomocysteïnemie inderdaad te berusten op een verlaagde activiteit van dit enzym.

Het vóórkomen van twee erfelijke risicofactoren voor hart- en vaatziekten,

namelijk milde hyperhomocysteïnemie en proteine (= eiwit) C tekort type 1, binnen één familie is in **Hoofdstuk 6** beschreven. Alleen bij die familieleden bij wie de twee risicofactoren gecombineerd voorkwamen, openbaarden zich hart- en vaatziekten. Op basis hiervan is een onderling versterkende werking van deze risicofactoren gesuggereerd.

Het homocysteïne-verlagende effect van vitamine behandeling is beschreven in Deel 2, Hoofdstuk 7 tot 9.

Allereerst wordt in **Hoofdstuk 7** een overzicht gepresenteerd van beschikbare gegevens over homocysteïne verlagende therapieën. Een verhoogd nuchter homocysteïne-gehalte kan met 40% tot 50% verlaagd worden door toediening van respectievelijk 0,65 mg of 5 mg foliumzuur in tabletvorm. Een hogere dosering van foliumzuur tot 10 mg dagelijks heeft geen verder homocysteïne-verlagend effect. Het verhoogde homocysteïne-gehalte na methionine belasting kan bij gebruik van 100 mg tot 250 mg vitamine B_6 40% dalen. Combinatie therapie van 100 mg vitamine B_6 en 5 mg foliumzuur geeft zelfs een halvering van het homocysteïne-gehalte na methionine belasting.

Het onderzoek beschreven in **Hoofdstuk 8** laat zien hoe vaak milde hyperhomocysteïnemie na methionine belasting voorkomt bij patiënten met een herseninfarct, hartinfarct of "etalageziekte" (dit is vaatverkalking in de slagaders naar of in de benen, in het proefschrift "perifere arteriosclerose" genoemd) en welk effect vitamine B_6 als homocysteïne-verlagende therapie heeft op de verhoogde homocysteïne-spiegels. De cijfers komen overeen met de in de literatuur gepresenteerde cijfers. In de periode van januari 1980 tot december 1990 zijn 421 patiënten, jonger dan 55 jaar, met een klinisch vastgelegd herseninfarct of "etalageziekte", onderzocht op milde hyperhomocysteïnemie door middel van de methionine belasting test in het Academisch Ziekenhuis Nijmegen. Bij 33% van de patiënten met "etalageziekte" en 20% van de patiënten met herseninfarct werd milde hyperhomocysteïnemie gevonden. Deze patiënten met een verhoogd homocysteïne- gehalte zijn vervolgens behandeld met vitamine B_6, in een dosering van 250 mg per dag gedurende 6 weken. Het verhoogde homocysteïne-gehalte na methionine belasting kon genormaliseerd worden bij 56% van de behandelde patienten.

In **Hoofdstuk 9** zijn 309 patiënten met "etalageziekte", hersen- of hartinfarct onderzocht in het Vrije Universiteit Ziekenhuis te Amsterdam op het voorkomen van milde hyperhomocysteïnemie, zowel nuchter als na methionine belasting. Bij 23% van de patiënten werd milde hyperhomocysteïnemie na methionine belasting vastgesteld, waarvan ongeveer de helft ook een nuchter verhoogd homocysteïne-gehalte liet zien. Het verhoogde (nuchtere en/of na methionine belasting) homocysteïne-gehalte in bloed kon bij meer dan 90% van de hyperhomocysteïnemische patiënten genormaliseerd worden door gecombineerde therapie met 250 mg vitamine B_6 en 5 mg foliumzuur. Op basis van de gegevens beschreven in **Hoofdstuk 8 en 9** kon geconcludeerd worden dat milde hyperhomocysteïnemie een veel voorkomende risicofactor voor vaatverkalkingen is, welke eenvoudig, veilig en goedkoop te behandelen is.

Aan negen homozygoten voor homocystinurie (patiënten met een ernstig

verhoogde homocysteïne-concentratie in bloed) is vitamine B_1 toegediend om de afbraak van methionine via de zogeheten "transamineringsroute" te stimuleren (**Hoofdstuk 10**). Deze patiënten hadden, ondanks hun homocysteïne-verlagende therapie, nog steeds een verhoogde homocysteïne-concentratie in het bloed. Indien ten gevolge van een behandeling met vitamine B_1 de homocysteïne-concentratie zou normaliseren bij deze groep van patiënten, dan zou vitamine B_1 mogelijk ook effectief kunnen zijn bij die patiënten met milde hyperhomocysteïnemie die geen normalisering van het homocysteïne-gehalte in het bloed ondanks gebruik van vitamine B_6 en foliumzuur laten zien. Echter, vitamine B_1 verlaagde de homocysteïne- concentratie bij acht van negen patiënten niet, en heeft dus geen betekenis als alternatieve behandeling bij patiënten met milde hyperhomocysteïnemie.

In Deel 3 van dit proefschrift, Hoofdstuk 11 tot 13, zijn enkele aspecten van het klinisch effect van homocysteïne-verlagende therapie beschreven.

Het effect op de vaatwandfunctie van behandeling met vitamine B_6 en foliumzuurtherapie gedurende één jaar bij patiënten met perifere arteriosclerose en mild hyperhomocysteïnemie, werd getest aan de hand van de bepaling van een tweetal indicatoren van de vaatwandfunctie, namelijk "von Willebrand factor" en "thrombomoduline" (**Hoofdstuk 11**). Deze waren vóór de start van de behandeling bij deze patiënten gestoord. Hiermee kon een verbetering van de vaatwandfunctie worden vastgesteld. Een andere indicator van de vaatwandfunctie, namelijk "weefsel-type plasminogeen activator", was vóór en na behandeling normaal.

De lichaamsslagader (= aorta) in de buik en ter hoogte van het begin van de slagaders naar de benen toe, zijn onderzocht met behulp van drie soorten opname technieken namelijk met twee soorten Magnetische Resonantie technieken (namelijk met Magnetic Resonance Imaging = MRI en met Magnetic Resonance Angiography = MRA) en met slagaderlijk vaatonderzoek door middel van inspuiten van contrastvloeistof (Digital Subtraction Angiography = DSA) bij 11 patiënten met klinisch bewezen arteriosclerose en milde hyperhomocysteïnemie (**Hoofdstuk 12**). Zes patiënten lieten vaatverkalking in één of meer slagaderlijke segmenten zien onderzocht door middel van DSA. Tachtig en 64% van de slagaderlijke segmenten werden respectievelijk met de MRI- en MRA-technieken in vergelijking met DSA correct beoordeeld. De gevoeligheid, de specificiteit en de nauwkeurigheid van de MRI-techniek waren respectievelijk 67%, 86% en 80%. Met MRA werden meestal slechtere resultaten verkregen. Geconcludeerd werd dat vaatverkalkingen diffuus verspreid aanwezig zijn bij patiënten met milde hyperhomocysteïnemie en dat de MRI-techniek een acceptabele methode is ter vaststelling van zelfs geringe arteriosclerose. Door deze studie kon MRI in de opgezette prospectieve studie als één van de methoden geïntroduceerd worden om progressie of regressie van de vaatverkalkingen in de vaatwand van hyperhomocysteïnemische patiënten te detecteren.

In een twee-jaar-durend onderzoek, waarbij de helft van de patiënten homocysteïne-verlagende therapie en de andere helft tabletten kreeg zonder B-vitaminen (placebo's) en waarbij zowel de patiënt als de arts niet wist welke behandeling de patiënt gebruikte (dubbel-blind), is het klinisch effect van gecombineerde vitamine B_6 en foliumzuurbehandeling beschreven (**Hoofdstuk 13**). Drie

van de zes placebo-behandelde patiënten en slechts één van de acht vitamine-behandelde patiënten lieten verergering van de vaatverkalkingen zien na twee jaar dagelijks gebruik van respectievelijk placebo of gecombineerde vitamine B_6 en foliumzuurtherapie. Bovendien liet één van de zes placebo-behandelde en vier van de acht vitamine behandelde patiënten een teruggang van de vaatverkalkingen zien. Hoewel het aantal bestudeerde patiënten klein is, kon een significant gunstig effect van de homocysteïne-verlagende therapie vastgesteld worden.

In **Hoofdstuk 14** wordt besproken in hoeverre aan de in de inleiding van dit proefschrift omschreven doelstellingen is voldaan. De resultaten van de onderzoeken in dit proefschrift worden vergeleken met die van andere onderzoekers. Ook worden suggesties gedaan voor toekomstig onderzoek.

Dankwoord

Nu het proefschrift afgerond is, ben ik me er meer dan ooit van bewust dat ik deze klus nooit alleen had kunnen klaren. Vanaf het begin van mijn onderzoek heb ik gezegd dat het onderwerp hyperhomocysteïnemie net een trein is die (toen) nog langzaam reed, maar al snel zijn vaart zou gaan verhogen. Heden ten dage blijkt het onderwerp "hyperhomocysteïnemie" een hogesnelheidstrein geworden te zijn, hetgeen alleen mogelijk is geworden doordat er zich zoveel wetenschappers met dit onderwerp bezighouden. Iedereen die direct of indirect betrokken is geweest bij de totstandkoming van dit proefschrift wil ik van harte danken. Een aantal van hen wil ik met name noemen. Zoals het hoort, staat dit dankwoord achterin het proefschrift, maar troost U, op de stellingen na wordt dit "hoofdstuk" het best gelezen.

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Prof.dr. J.H.J. Ruijs. Het verrichten van wetenschappelijk onderzoek is vaak een kwestie van een lange adem. U heeft mij mijn gang laten gaan, waarvoor ik U hartelijk dank.

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A. Garretsen - van Loon, I. Konijnenberg - Kramer en S. Vloet. Beste Anne-Marie, Inge en Stephanie, jullie hebben samen circa 6.000 patiënten gezien, 9.000 methionine belastingtesten uitgevoerd, 17.900 bloedmonsters afgedraaid en circa 60 kilogram methionine is door jullie handen gegaan. Een ieder die dit leest weet dat jullie dus onmisbaar waren voor het onderzoek.

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A.M.T. Engbersen, beste Astrid, jij hebt je nauwgezet bezig gehouden met

de "thermolabielen" onder ons. (Of elke contrôle persoon daar zo blij mee was ...?). Dankzij jou kwam hoofdstuk 5 tot stand, waarvoor ik je hartelijk dank.

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Gaarne wil ik tevens Addy de Graaf - Hess, Dinny Oppenraaij - Emmerzaal, Henriëtte van Lith - Zanders, Erik Stevens, José Haenen, Adriënne van Maarseveen, Nathalie van der Put en Jolanda van Nieuwkerk - Remmits hartelijk danken voor hun inzet.

Het proefschrift is een verlengstuk van een lange opleiding en leerperiode. De basis hiervoor werd gelegd door mijn ouders, die door hun methode van opvoeding mij een solide en stabiele ondergrond hebben meegegeven. Ik profiteer hier nog dagelijks van en ben jullie veel dank verschuldigd.

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Curriculum vitae

De schrijfster van dit proefschrift werd geboren op 1 april 1965 te Son c.a. In 1983 behaalde zij het VWO-diploma aan het van der Putt-lyceum te Eindhoven. Aansluitend werd aangevangen met de studie geneeskunde aan de Katholieke Universiteit te Nijmegen, alwaar zij haar arts-diploma in september 1991 ontving. Sedert juni 1991 verrichtte zij op het laboratorium kindergeneeskunde en neurologie (hoofd: prof. dr. ir. J.M.F. Trijbels), de afdeling endocriene ziekten (hoofd: prof. dr. P.W.C. Kloppenborg) en de afdeling radiologie (hoofd: prof. dr. J.H.J. Ruijs) van het Academisch Ziekenhuis Nijmegen als arts-onderzoeker het wetenschappelijk onderzoek waarvan de resultaten zijn beschreven in dit proefschrift. In juni 1993 is zij begonnen met haar opleiding tot radiologe in het Academisch Ziekenhuis Nijmegen (opleider: prof. dr. J.H.J. Ruijs).

Stellingen

1 Milde hyperhomocysteinemie is een veel voorkomende risicofactor voor arteriosclerose in patienten met claudicatio intermittens of herseninfarct vóór hun vijftigste levensjaar
Dit proefschrift.

2. Milde hyperhomocysteinemie leidt tot gegeneraliseerde arteriosclerose
Dit proefschrift.

3 Milde hyperhomocysteinemie na een gestandaardiseerde methionine belastingtest gaat in circa 50% niet gepaard met een verhoogd homocysteine gehalte in nuchter bloed.
Dit proefschrift.

4 Milde hyperhomocysteinemie, zowel nuchter als na een methionine belastingtest, komt in respectievelijk 21% en 32% voor bij familieleden van hyperhomocysteinemische patienten met arteriosclerose
Dit proefschrift.

5 Homozygotie voor thermolabiel 5,10-methyleentetrahydrofolaat reductase deficientie is een veel voorkomende genetische oorzaak van milde hyperhomocysteinemie.
Dit proefschrift.

6 De combinatie van behandeling met vitamine B_6 en foliumzuur kan in vrijwel alle hyperhomocysteinemische patienten met arteriosclerose het verhoogde homocysteine-gehalte in het bloed normaliseren.
Dit proefschrift.

7 Vitamine B_6 en foliumzuur therapie bij hyperhomocysteinemische patienten met arteriosclerose lijkt progressie van deze vaatverkalkingen tegen te kunnen gaan.
Dit proefschrift.

8 Een mens is pas oud als hij/zij zegt de leeftijd te hebben die hij/zij dit jaar nog moet bereiken.

9. De vervanging van de postzegel door een stempel is een uiting van cultuurverarming

10 Het gras groeit niet harden door er aan te trekken.

11 Een appel die niet ver van de boom valt, heeft geen groei mogelijkheden

12 Mensen die van lange nagels en lang haar houden, zijn necrofielen.

13 Als een inspreekapparaat een antwoordapparaat of telefonische beantwoorder zou zijn, zou er niet zo vaak teruggebeld hoeven worden

14 Gepromoveerde deskundigen dienen de bevoegdheid te kunnen verkrijgen bij een promotie als promotor te mogen optreden.